UNIVERSIDADE DE SÃO PAULO
Faculdade de Ciências Farmacêuticas

Produtos de origem microbiana de interesse farmacêutico, alimentar e ambiental

Ricardo Pinheiro de Souza Oliveira

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SUMÁRIO

LISTA DE ABREVIATURAS E SIGLAS

BAL	Bactérias Ácido-Láticas
BLIS	Bacteriocinas
BSM	*Bifidus Seletive Medium*
CMC	Concentração Micelar Crítica
FAO/WHO	Food and Agriculture Organization/World Health Organization
FDA	American Food and Drug Administration
FI	Fator de indução
GRAS	Generally Recognized as Safe
HeO	Heterofermentativos Obrigatórios
HoO	Homofermentativos Obrigatórios
MHK	Membrana Histidina Quinase
MRS	*Man, Rogosa e Sharpe*
PFL	Piruvato Formato Liase
Phe	Metabolismo da Fenilalanina
PLA	Ácido Fenilático
RR	Regulador de Resposta
SLC	Sobrenadante Livre de Células
SM	Leite Desnatado
TS	Tensão Superficial
USP	Universidade de São Paulo
YE	Extrato de Levedura

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LISTA DE QUADROS

OLIVEIRA, R.P.S. Produtos de origem microbiana de interesse farmacêutico, alimentar e ambiental. 2017. 230 f. Tese (Livre-Docência) – Faculdade de Ciências Farmacêticas, Universidade de São Paulo, São Paulo, 2017.

RESUMO

Os micro-organismos produzem um conjunto de sistemas de defesa, incluindo produtos metabólicos, como ácido lático, peptídeos antimicrobianos (bacteriocinas) e biossurfactantes. Nesse texto sistematizado estão resumidas as minhas principais contribuições científicas com relação à importância dessas biomoléculas no campo farmacêutico, alimentar e ambiental. O critério utilizado para a escolha desse compilado de resultados foi selecionar apenas os artigos publicados ou aceitos para publicação, dos quais sou coordenador, autor correspondente ou coautor. Os resultados dos artigos submetidos não serão apresentados nesta versão, mas sim comentados na seção “conclusões e perspectivas”. Inicialmente, são discutidos resultados referentes à produção biotecnológica de ácido lático e bacteriocinas utilizando resíduos agroindustriais e fontes alternativas de carbono e nitrogênio. Fundamentos bioquímicos e metabólicos da fermentação por bactérias ácido-láticas, bem como biossíntese, mecanismo de ação e aplicação de diferentes bacteriocinas também são abordados neste trabalho. No que tange aos biossurfactantes, se estudou influência do tolueno e da salinidade na produção biotecnológica destes compostos anfifílicos. Ademais, os efeitos da temperatura, do pH e de diferentes substratos na produção de biossurfactantes também foram abordados. Por fim, são explanadas as novas frentes de trabalho do meu grupo de pesquisa.

Palavras-chave: Cultivo microbiano, biomoléculas, ácido lático, bacteriocinas, biossurfactantes.

OLIVEIRA, R.P.S. Products of Microbial Origin to Pharmaceutical, Food and Environmental applications. 2017. 230 f. Tese (Livre-Docência) – Faculdade de Ciências Farmacêticas, Universidade de São Paulo, São Paulo, 2017.

ABSTRACT

Microorganisms produce a set of defense systems, including metabolic products such as lactic acid, antimicrobial peptides (bacteriocins) and biosurfactants. In this systematized text, I have summarized my main scientific contributions regarding the importance of these biomolecules in pharmaceutical, food and environmental fields. The criterion used to choose these compiled results was to only select articles published or accepted for publication which I am either coordinator, corresponding author or co-author. The results of submitted articles will not be presented in this version, but they will be commented in the section "conclusions and perspectives". Initially, there is the discussion of results concerning biotechnological production of lactic acid and bacteriocins using agroindustrial residues as alternative sources of carbon and nitrogen. In this work, I have also addressed biochemical and metabolic bases of fermentation by acid lactic bacteria, biosynthesis, as well as mechanisms of action and application of different bacteriocins. In regard to biosurfactants, we researched the influence of toluene and salinity on the biotechnological production of these amphiphilic compounds. In addition, there are studies of the effects of temperature, pH and different substrates in the production of biosurfactants. Finally, new research line groups are explained.

Keywords: Microbial cultivation, biomolecules, lactic acid, bacteriocins, biosurfactants.

1. INTRODUÇÃO

Os micro-organismos produzem um conjunto de sistemas de autodefesa, incluindo os antibióticos, subprodutos metabólicos, como ácido lático produzido por espécies de lactobacilos, agentes líticos tais como lisozimas, diferentes exotoxinas proteicas, peptídeos antimicrobianos (bacteriocinas) e substâncias inibitórias semelhantes às bacteriocinas (BLIS) (Cavera *et al*., 2015). Este arsenal biológico é notável não só na sua diversidade, mas também na sua abundância natural.

O ácido lático, além de ser considerado um composto antimicrobiano, possui diversas aplicações nas indústrias farmacêutica, cosmética e alimentar. Neste contexto, nosso grupo de pesquisa relatou os processos fermentativos e biotecnológicos para a produção de ácido lático (**APÊNDICE 1**). Em particular, na indústria cosmética, o ácido lático é utilizado na manufatura de produtos de higiene e estética, devido aos seus efeitos hidratante, antimicrobiano e rejuvenescedor na pele, bem como de produtos de higiene bucal. Na indústria farmacêutica, o ácido lático é utilizado como suplemento na formulação de fármacos dermatológicos e contra a osteoporose.

Aproximadamente 70% do ácido lático produzido são utilizados na indústria de alimentos devido ao seu papel na produção de derivados lácteos, como iogurtes e queijos. Na preparação de iogurtes é o principal produto da cofermentação de *Streptococcus thermophilus* e *Lactobacillus bulgaricus*. Na fabricação de queijos, o decréscimo do pH consequente à liberação de ácido lático desencadeia a agregação de micelas de caseína. Por vezes, dependendo das características sensoriais desejadas no produto final, a acidificação direta com ácido lático é explorada para evitar o risco de proliferação de micro-organismos indesejáveis. No campo da produção de grãos e silagem, o ácido lático se forma espontaneamente devido à presença de micro-organismos que realizam a fermentação lática da matéria-prima,

alterando assim o aroma, além de impedir o crescimento de bactérias patogênicas.

As bacteriocinas são definidas como porções proteicas biologicamente ativas com um modo de ação bactericida ou bacteriostático (Riley; Wertz, 2002; Collins *et al*., 2010). A família inclui uma diversidade de proteínas em termos de tamanho (Riley; Gordon, 1999) e os efeitos resultantes de suas interações com as bactérias-alvo consiste em uma área de estudo que vem atraindo muitos pesquisadores.

Em geral, os testes de atividade antimicrobiana utilizando métodos de difusão em ágar não distinguem as atividades inibitórias das bacteriocinas em comparação com agentes antimicrobianos, como bacteriófagos, metabólitos primários (água oxigenada, ácido láctico entre outros) ou antibióticos sintetizados não ribosomicamente (bacitracina). Tais testes também não discriminam a inibição atribuída à depleção de nutrientes ou às atividades combinadas de múltiplas bacteriocinas e/ou outros agentes inibitórios. Para isso, é recomendado o uso do acrônimo BLIS (para substâncias inibitórias semelhantes às bacteriocinas) para se referir a agentes inibidores ainda não caracterizados "semelhantes às bacteriocinas" na sua atividade antimicrobiana.

Esses compostos antimicrobianos são produzidos principalmente por bactérias Gram-positivas e Gram-negativas e, mais recentemente, foram descritos em alguns membros do grupo *Archaea* (Ellen *et al*., 2011). Esses compostos antimicrobianos apresentam espectro de ação contra uma variedade de micro-organismos incluindo bactérias Gram-positivas e Gram-negativas, protozoários, fungos e vírus (Reddy *et al*., 2004).

As bacteriocinas produzidas por bactérias Gram-negativas são diversas. Em *Escherichia coli*, mais de 30 bacteriocinas foram identificadas e, indubitavelmente, muitas delas ainda não foram descobertas. A diversidade

presente em outras espécies Gram-negativas, incluindo outros membros das *Enterobacteriaceae*, ainda é pouco explorada (Gordon *et al*., 2007).

Já as bacteriocinas produzidas por bactérias Gram-positivas são abundantes e mais diversificadas do que as encontradas em bactérias Gram-negativas. Os peptídeos antimicrobianos produzidos por bactérias Gram-positivas, particularmente por bactérias ácido-láticas, apresentam amplo espectro como bioconservante de alimentos e como agentes terapêuticos (Jack *et al*., 1995; Cotter *et al*., 2005; Galvez *et al*., 2008).

2. BACTÉRIAS ÁCIDO-LÁTICAS

No início do século XX, o termo “bactérias ácido-láticas” foi utilizado para se referir aos micro-organismos do leite, mas similaridades entre estes com outras bactérias produtoras de ácido lático também foram observadas em 1919 por Orla-Jensen (1919), formando, portanto, a base da presente classificação das bactérias láticas (Von Wright; Axelsson, 2012).

2.1 Posição taxonômica das bactérias láticas

Do ponto de vista taxonômico, as bactérias ácido-láticas (BAL) constituem um grupo de bactérias Gram-positivas, tendo como principal produto metabólico o ácido lático, provindo da fermentação de carboidratos (Mayo *et al*., 2010). Esses micro-organismos são catalase-negativos, não esporulados, ácido-tolerantes e anaeróbicos facultativos. Estes micro-organismos são desprovidos de citocromos e podem assumir a forma de cocos ou bastonetes. Exceto para algumas espécies, as BAL são consideradas não patogênicas e reconhecidas como seguras *Generally Recognized as Safe* (GRAS) (Von Wright; Axelsson, 2012).

Estes micro-organismos pertencem ao filo *Firmicutes*, classe *Bacilli*, e ordem *Lactobacillales*. As diferentes famílias incluem *Aerococcaceae*, *Carnobacteriaceae*, *Enterococcaceae*, *Lactobacillaceae*, *Leuconostocaceae* e *Streptococcaceae*. Os gêneros comuns e suas principais características são listados no **QUADRO 1.**

Quadro 1. Gêneros de BAL e suas características.

FAMÍLIA	GÊNERO	Forma	CO_2 (Glicose)	Crescimento (10ºC)	Crescimento (45ºC)	Crescimento (6,5% de NaCl)	Crescimento (18% de NaCl)	Crescimento (pH 4,4)	Crescimento (pH 9,6)	Ácido lático
Aerococcaceae	*Aerococcus*	Cocos (tétrades)	-	+	-	+	-	-	+	L
Carnobacteriaceae	*Carnobacterium*	Bastonetes	-	+	-	ND	-	ND	-	L
Enterococcaceae	*Enterococcus*	Cocos	-	+	+	+	-	+	+	L
	Tetrageonococcus	Cocos (tétrades)	-	+	-	+	+	-	+	L
	Vagonococcus	Cocos	-	+	-	-	-	Variável	-	L
Lactobacillaceae	*Lactobacillus*	Bastonetes	Variável	Variável	Variável	Variável	-	Variável	-	D, L, DL
	Pediococcus	Cocos (tétrades)	-	Variável	Variável	Variável	-	+	-	L, DL
Leuconostocaceae	*Leuconostoc*	Cocos	+	+	-	Variável	-	Variável	-	D
	Oenococcus	Cocos	+	+	-	Variável	-	Variável	-	D
	Weissella	Cocos*	+	+	-	Variável	-	Variável	-	D, DL
Streptococcaceae	*Lactococcus*	Cocos	-	+	-	-	-	Variável	-	L
	Streptococcus	Cocos	-	-	Variável	-	-	-	-	L

Fonte: Adaptado de von Wright e Axelsson (2012).

2.2 Fundamentos bioquímicos e metabólicos da fermentação lática por bactérias

O gênero mais importante e heterogêneo de bactérias láticas é o *Lactobacillus*, que inclui espécies com propriedades bioquímicas e fisiológicas muito diferentes. Destaca-se por suas vantagens no que diz respeito à elevada velocidade de crescimento e produtividade (Kylä-Nikkilä *et al*., 2000). Os micro-organismos pertencentes a este gênero podem utilizar principalmente duas vias fermentativas para o consumo de glicose (homofermentativa e heterofermentativa). Dependendo do micro-organismo é possível de se fermentar açúcares de diferentes procedências (**QUADRO 2**).

Quadro 2. Espécies de BAL e as diferentes matérias-primas empregadas no cultivo.

Material	Micro-organismo	Fonte de carbono
Monossacarídeos e dissacarídeos		
Melaço	*L. casei*	Sacarose
	L. lactis	Sacarose
Xarope de abacaxi	*L. lactis*	Sacarose
Leite de camelo	*L. delbruecki*	Lactose
Leite de vaca	*L. delbruecki*	Lactose
Soros lácteos	*L. acidophilus*	Lactose
	L. bulgaricus	Lactose
	L. delbruecki	Lactose
	L. casei	Lactose
	L. helveticus	Lactose
	Lactococcus lactis	Lactose
	S. thermophilus	Lactose
Suco de tâmara	*L. rhamnosus*	Sacarose
Materiais amiláceos		
Milho	*L. amylophilus*	Amido
Batata	*L. amylophilus*	Amido
	L. delbrueckii	Glicose[a]
Trigo	*L. amylophilus*	Amido
	L. bulgaricus	Glicose[a]
	L. casei	Glicose[a]
	L. lactis	Glicose[a]
Arroz	*L. delbrueckii*	Glicose[a]
Cevada	*L. casei*	Glicose[a]
Yucca	*L. lactis*	Glicose[a]
	L. plantarum	Amido
	L. delbruecki	Glicose[a]
	L. casei	Glicose[a]
Tapioca	*L. plantarum*	Glicose[a]
Hidrolisados lignocelulósicos		
Bambú	*L. plantarum*	Glicose
Cartón corrugado	*L. coryniformis*	Glicose
Fibra de alfafa	*L. delbrueckii*	Glicose
	L. pentoaceticus	Glicose
	L. plantarum	Glicose
	L. xylosus	Glicose
Fibra de soja	*L. delbrueckii*	Glicose
	L. plantarum	Glicose
Madeira de eucalipto	*L. delbrueckii*	Glicose
Bagaço de uva	*L. pentosus*	Xilose
Palha de trigo	*L. pentosus*	Xilose
	L. brevis	Xilose
Papel residual	*L. rhamnosus*	G/X/C
Polpa de celulose	*L. delbruecki*	Glicose
Resíduo celulósico	*L. casei*	Glicose
RSU[b]	*L. pentosus*	X/G/A
	L. plantarum	X/G/A
Bagaço de uva	*L. pentosus*	X/G/A
		Glicose
Espiga de milho	*L. delbrium*	Glicose

[a]Hidrolisados de amido; [b]Residuos urbanos; X = xilose/G = glicose/A = arabinose/C = celobiose; *L.* = *Lactobacillus*.

Fonte: Adaptado de Martinez *et al.,* 2013a.

2.3 Fermentação homolática

Este processo se desenvolve em duas etapas. Na primeira etapa, denominada glicólise ou rota de Embden-Meyerhoff-Parnas, a glicose se transforma em ácido pirúvico. Na segunda etapa o ácido pirúvico se reduz a ácido lático empregando o NADH produzido na primeira etapa. Desta forma, a partir de glicose se obtêm o ácido lático como único produto (**FIGURA 1**) segundo a reação global:

Glicose → 2 Ácido lático + 2 ATP

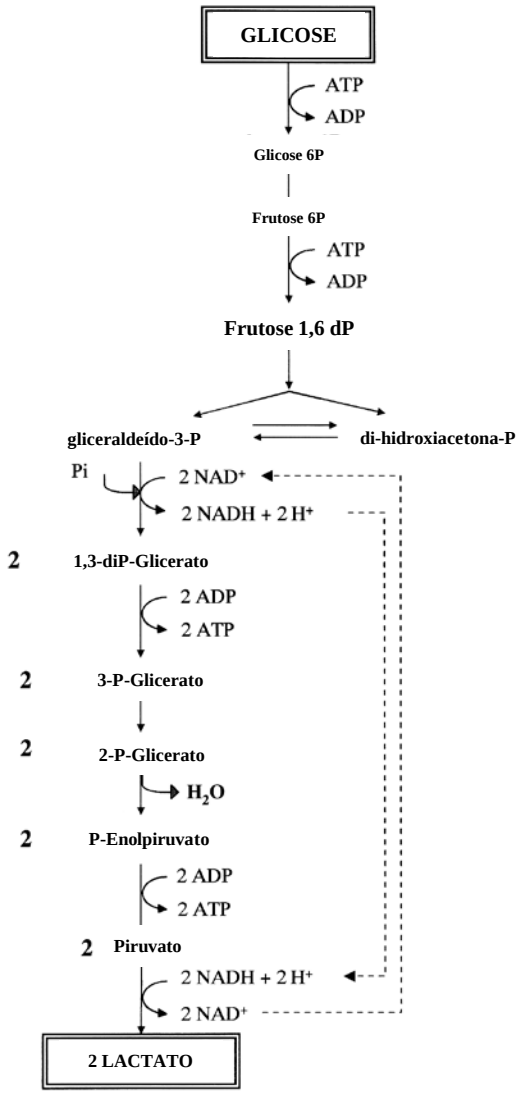


Figura 1. Fermentação homolática da glicose.

Fonte: Adaptado de Axelsson, 2004 e Mayo *et al*. 2010.

Os micro-organismos que somente utilizam esta rota para o consumo dos carboidratos recebem o nome de Homofermentativos Obrigatórios (HoO) e entre eles se encontram *L. acidophilus, L. amylophilus, L. bulgaricus, L. helveticus* e *L. salivarius* (Nigatu, 2000; Sanders; Klaenhammer, 2001).

Na fermentação homolática se obtêm 2 moles de ácido lático por cada mol de glicose, com um rendimento teórico de 1 g de produto por g de substrato, enquanto os rendimentos experimentais são inferiores (0,74-0,99 g/g) devido a que parte da fonte de carbono se emprega na formação de 0,07-0,22 g/g de biomassa (Srivastava *et al*., 1992; Hofvendahl; Hahn-Hägerdal, 1997; Bruno-Bárcena *et al*., 1999; Burgos-Rubio *et al*., 2000). Em condições de limitação da fonte de carbono, presença de fontes de carbono diferentes da glicose, valores altos de pH ou baixas temperaturas, alguns micro-organismos homofermentativos podem gerar ácido fórmico por fermentação ácido-mista (Hofvendahl; Hähn-Hägerdal, 2000) mediante a ação da piruvato formato liase (PFL).

2.4 Fermentação heterolática

Este processo se caracteriza pela formação do ácido lático juntamente com outros produtos (CO_2, etanol e/ou ácido acético) (**FIGURA 2**).

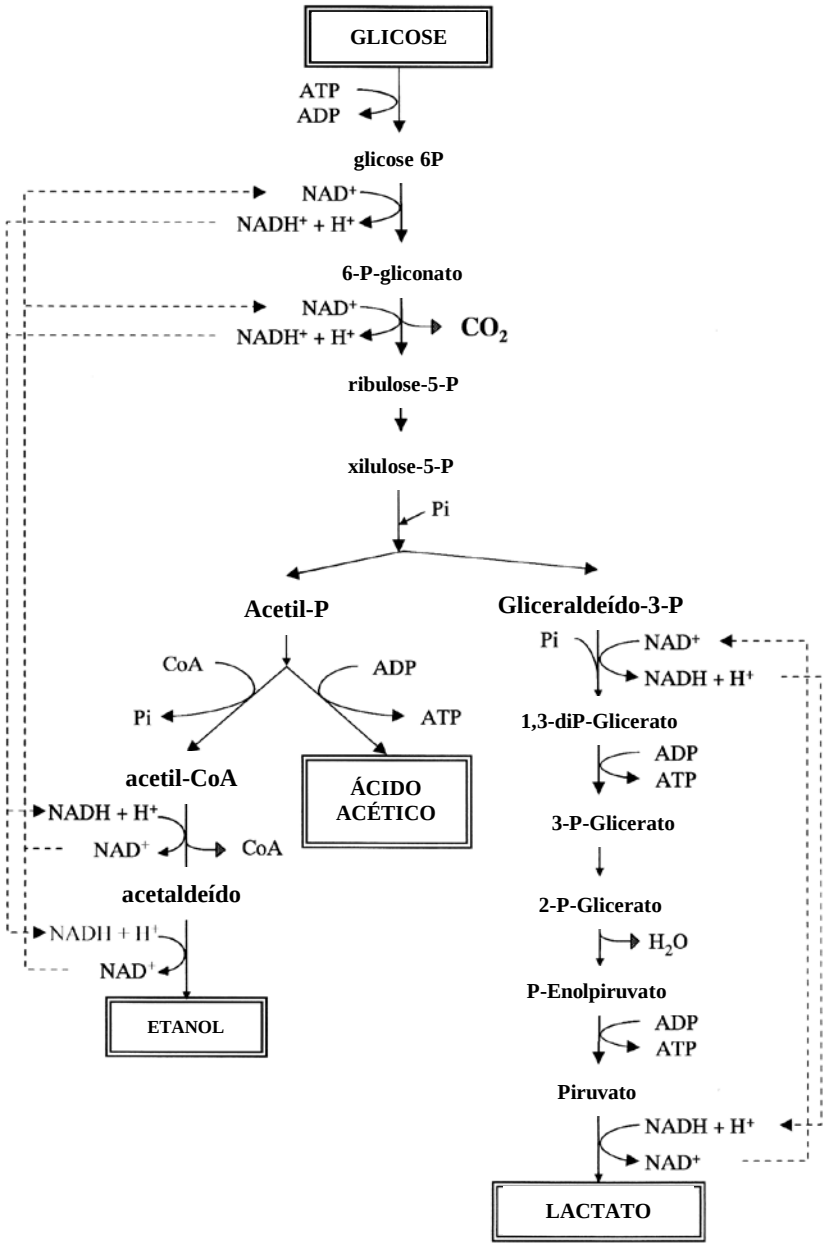


Figura 2. Fermentação heterolática da glicose.

Fonte: Adaptado de Axelsson, 2004 e Mayo *et al*., 2010.

A primeira etapa de degradação da glicose recebe o nome de rota das pentoses-fosfato, onde se obtém o gliceraldeído 3-fosfato, acetilfosfato e CO_2. O gliceraldeído 3-fosfato entra na glicólise e, através da fermentação lática, se transforma em ácido lático.

O acetilfosfato se transforma em ácido acético e/ou etanol através das seguintes reações:

Glicose → Ácido lático + CO_2 + Etanol + ATP

Glicose → Ácido lático + CO_2 + Ácido acético + 2 ATP + 2 NADH

A relação entre as quantidades de ácido acético e etanol, que reduzem o rendimento teórico a 0,50 g/g, depende da capacidade do micro-organismo em regenerar o NADH obtido nas primeiras etapas do processo e também de suas necessidades energéticas.

Os micro-organismos que somente utilizam esta via metabólica para o consumo dos carboidratos recebem o nome de *Heterofermentativos Obrigatórios* (HeO), dentre os quais se encontram *L. brevis, L. fermentum, L. parabuchneri* e *L. reuteri* (Nigatu, 2000; Sanders; Klaenhammer, 2001).

Em teoria, qualquer fonte de carboidrato contendo pentoses ou hexoses poderia ser utilizada para a produção de ácido lático. Neste aspecto, o nosso grupo de pesquisa publicou um trabalho (**APÊNDICE 1**) em que foram exploradas possíveis fontes de carbono, como os subprodutos da indústria alimentar, tais como melaço e soro de leite. O melaço possui elevado teor de sacarose, além de ser barato e abundante, enquanto o soro de leite, cuja eliminação constitui um desafio ambiental grave, tem elevado teor de lactose. Os substratos poliméricos também foram explorados nessa pesquisa. De fato, estes substratos contêm polissacarídeos que, na maioria dos casos, não são assimilados diretamente pelos micro-organismos, sendo, portanto, hidrolisados em uma etapa anterior. Nesse caso, englobam-se os materiais amiláceos e os compostos hemicelulósicos.

Esse levantamento científico permitiu ao nosso grupo de pesquisa explorar diferentes substratos para obtenção de biomoléculas de alto valor agregado, como o ácido lático, bacteriocinas e biossurfactantes.

3. BACTERIOCINAS

O termo antibiótico é, genericamente, usado para descrever substâncias produzidas por organismos, que interferem seletivamente no crescimento de outros organismos. Dentro dessa categoria extremamente ampla de moléculas bioativas, o subconjunto conhecido como bacteriocinas compreende os compostos proteicos sintetizados nos ribossomos e liberados extracelularmente por bactérias, que podem interferir no crescimento de outras bactérias (Heng *et al*., 2007).

A primeira bacteriocina foi identificada por Gratia (1925) como sendo uma proteína antimicrobiana produzida por *Escherichia coli*, denominada colicina. Posteriormente, outros compostos antimicrobianos similares foram descritos para várias espécies bacterianas, não somente na família *Enterobacteriaceae*, mas também em outras. O termo bacteriocina foi proposto por Jacob e colaboradores (1952), o qual passou a ser utilizado mais frequentemente a partir dos estudos de Tagg e colaboradores (1976) e Klaenhammer (1988).

O interesse em bacteriocinas produzidas por micro-organismos GRAS, como as BAL, levou à descoberta da nisina, que é a única bacteriocina autorizada para uso como conservante de alimentos (Gharsallaoui *et al*., 2016). Este peptídeo antimicrobiano foi primeiramente comercializado na Inglaterra em 1953, tendo, desde então, sido aprovado para uso em mais de 48 países.

Notadamente, a nisina foi avaliada como sendo segura para uso alimentar pela Food and Agriculture Organization/World Health Organization (FAO/WHO) em 1969. Posteriormente, em 1983, esta bacteriocina foi considerada um aditivo alimentar pela União Européia (número E234) assim como, em 1988, foi aprovada pelo American Food and Drug Administration (FDA) para uso em queijos processados (Cotter *et al*., 2005). Já no Brasil, o uso da nisina passou a ser autorizado, em 1996, em queijos na concentração de até 12,5 mg/kg (Brasil. Ministério da Saúde, 1996). Outra bacteriocina que

possui grande importância no campo alimentar e farmacêutico é a pediocina. Esta biomolécula é muito conhecida devido à sua eficácia contra *Listeria monocytogenes*.

É importante destacar que as bacteriocinas vêm demostrando sua importância nas áreas alimentar e farmacêutica. Portanto, é de grande valia unir diferentes conhecimentos científicos para investigar a eficácia destes compostos antimicrobianos, já que há um aumento significativo de micro-organismos resistentes aos antibióticos.

3.1 Classificação das bacteriocinas

Existem diferentes classificações para as bacteriocinas, gerando o aparecimento de termos imprecisos como “verdadeiras bacteriocinas”, que englobariam as colicinas, as de *Lactobacillus* spp. e de outras BAL. O nosso grupo de pesquisa, através do trabalho de Balciunas e colaboradores (2013) (**APÊNDICE 2**) fez um levantamento sobre estas diferentes classificações. De acordo com Klaenhammer (1993), as bacteriocinas de bactérias Gram-positivas podem ser divididas em quatro classes.

A classe I dos lantibióticos, representada pela nisina, reúne peptídeos termostáveis de baixa massa molar (<5 kDa) caracterizados pela presença de lantionina e derivados. A classe II é composta por pequenos peptideos termostáveis (<10 kDa) divididos em três subclasses: IIa (pediocina e enterocina), IIb (lactocina G) e IIc (lactocina B). A classe III é representada por peptídeos termolábeis de elevada massa molar (> 30 kDa) tais como a helveticina J, enquanto que na classe IV encontram-se grandes peptídeos compostos de carboidratos ou lipídeos. No entanto, Cleveland e colaboradores (2001) acreditam que essas estruturas são artefatos de purificação parcial e não uma nova classe de bacteriocinas.

Cotter e colaboradores (2005) sugeriram uma nova classificação em que as bacteriocinas são divididas em duas categorias: lantibióticos ou bacteriocinas que contém lantionina (classe I) e os peptídeos antimicrobianos lineares não modificados (classe II). Drider e colaboradores (2006) dividiram as bacteriocinas em três grandes classes, de acordo com as características genéticas e bioquímicas dos micro-organismos produtores (**QUADRO 3**).

Quadro 3. Classificação de bacteriocinas de bactérias Gram-positivas de acordo com Drider *et al.*

Classificação	Características	Subcategoria	Exemplos
Classe I	Lantibióticos (contendo lantionina e β-lantionina	Tipo A: moléculas lineares (< 4kDa)	Nisina, subtilina, epidermina
		Tipo B: moléculas globulares (1,8 - 2,1kDa)	Mersacidina
Classe II	Peptídeos termoestáveis (<10kDa)	Subclasse IIa (antilistérica)	Pediocina, enterocina, sakacina
		Subclasse IIb (compostos de dois peptídeos)	Plantaricina
		Subclasse IIc (outras bacteriocinas)	Lactococina
Classe III	Peptídeos termolábeis (>30kDa)		Helveticina J, milericina B

Fonte: Adaptado de Drider *et al.,* 2006.

Os peptídeos da classe I são representados pelos lantibióticos, que são pequenos peptídeos modificados pós-tradução, que contêm aminoácidos incomuns, tais como a lantionina. A classe II inclui as bacteriocinas não modificadas, que são subdivididas em três subclasses, nomeadamente, a classe IIa (bacteriocinas do tipo pediocina), a classe IIb (bacteriocinas de dois peptídeos) e IIc (outras bacteriocinas). A designação de bacteriocinas do tipo pediocina refere-se à pediocina PA-1/AcH, que foi a primeira bacteriocina da classe IIa caracterizada (Biswas *et al*., 1991; Nieto Lozano *et al.,* 1992). Já os peptídeos da classe III são proteínas termossensíveis (Klaenhammer, 1993).

Recentemente, Cavera e colaboradores (2015) propuseram classificar as bacteriocinas de micro-organismos Gram-positivos, como as BAL, identificadas como da classe I, decorrentes de modificação pós-translacional, e, as da classe II, as quais podem apresentar pequenas ou nenhuma dessas modificações. Ademais, as bacteriocinas que são maiores do que 10 kDa são incluídas na classe III.

As bacteriocinas de bactérias Gram-negativas são divididas em pequenos peptídeos, como as microcinas (< 10kDa) e grandes peptídeos, como as colicinas (25-80kDa) (Chavan; Riley, 2007).

3.2 Biossíntese

A produção de bacteriocinas é desencadeada via "*quorum sensing*" por uma resposta ao aumento da densidade populacional ou por outro estresse ambiental (Cotter *et al*., 2005). Geralmente, estes peptídeos antimicrobianos são sintetizados na forma inativa devido à presença de uma sequência líder na sua porção N-terminal (**APÊNDICE 3**). A pré-bacteriocina é transportada até a membrana citoplasmática e sua sequencia líder é clivada por proteínas acessórias, sendo, assim, finalmente transportada para o meio extracelular via transportadores ABC específicos de bacteriocinas ou por sistema geral de excreção/secreção (*Sec)* (Nes *et al*., 1996; Cotter *et al*., 2005). No caso das pediocinas a sequência líder possui de 18 a 27 resíduos de aminoácidos com duas glicinas no final, que sinalizam a região de clivagem para posterior excreção realizada por transportadores ABC (Ray *et al*., 1999) (**FIGURA 3**).

Na **FIGURA 3**, o fator de indução é constantemente sintetizado e secretado pelas células produtoras (1). Em uma dada concentração no meio extracelular (2), o FI interage com o receptor MHK presente na própria célula e desencadeia a autofosforilação desta proteína de membrana (3). O resíduo de fosfato é transferido para o RR que ativa a expressão do(s) operon(s) relacionados às bacteriocinas da classe IIa (4). Este composto antimicrobiano é

sintetizado na forma de pré-peptideo (5) contendo uma sequência N-terminal que é clivada por proteínas assessórias e em seguida, a forma ativa da bacteriocina é secretada pelo sistema de transporte ABC. A interação das bacteriocinas deste grupo nas células alvo ocorre pela ligação dos receptores de membrana específicos ou por interação eletrostática com a membrana plasmática (6). Após esta interação, bacteriocinas da classe IIa se inserem na membrana formando poros, que por sua vez induzem a dissipação total ou parcial de $\Delta\Psi$ e ΔpH. As bacteriocinas da classe IIa também interagem com receptores específicos das células produtoras, entretanto, a presença da imunoproteína correspondente previne a formação de poros (7).

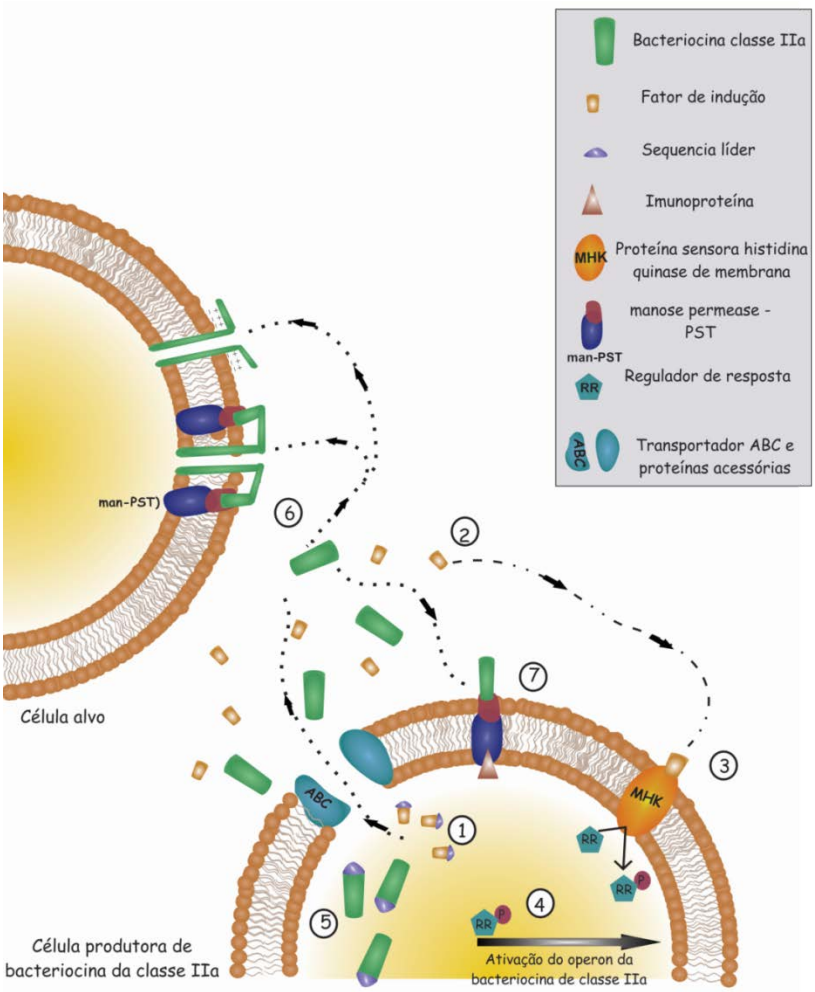


Figura 3. Biossíntese, regulação, autoimunidade e modo de ação de bacteriocinas da classe IIa.

Fonte: Adaptado de Porto *et al*., 2017.

Bacteriocinas da classe I sofrem modificações pós-traducionais por enzimas específicas ancoradas na membrana (NisB, NisC) antes de serem exportadas por transportadores ABC NisT (Lubelski *et al*., 2008) (**FIGURA 4**).

Na **FIGURA 4**, a nisina é sintetizada em sua forma inativa (1), contendo uma sequência líder N-terminal que é clivada por proteínas assessórias presentes na membrana. Posteriormente, a forma ativa desta bacteriocina é transportada para o meio extracelular via sistema tipo ABC (2). No meio externo a própria nisina interage com o receptor MHK da célula produtora e desencadeia a autofosforilação deste receptor (3). O resíduo de fosfato é transferido para o RR que ativa a expressão do(s) operon(s) relacionados à nisina (4), aumentando assim a produção desta bacteriocina (5). A nisina interage com o lipídeo tipo 2 na membrana plasmática da célula alvo e possui dois mecanismos de ação: i) formação de poros ii) Inibição da síntese de parede celular (6). A autoimunidade das células produtoras de nisina também ocorre por duas vias: i) Imunoproteína que impede a formação de poros ii) Retirada da nisina já inserida na membrana citoplasmática por transportadores específicos (7).

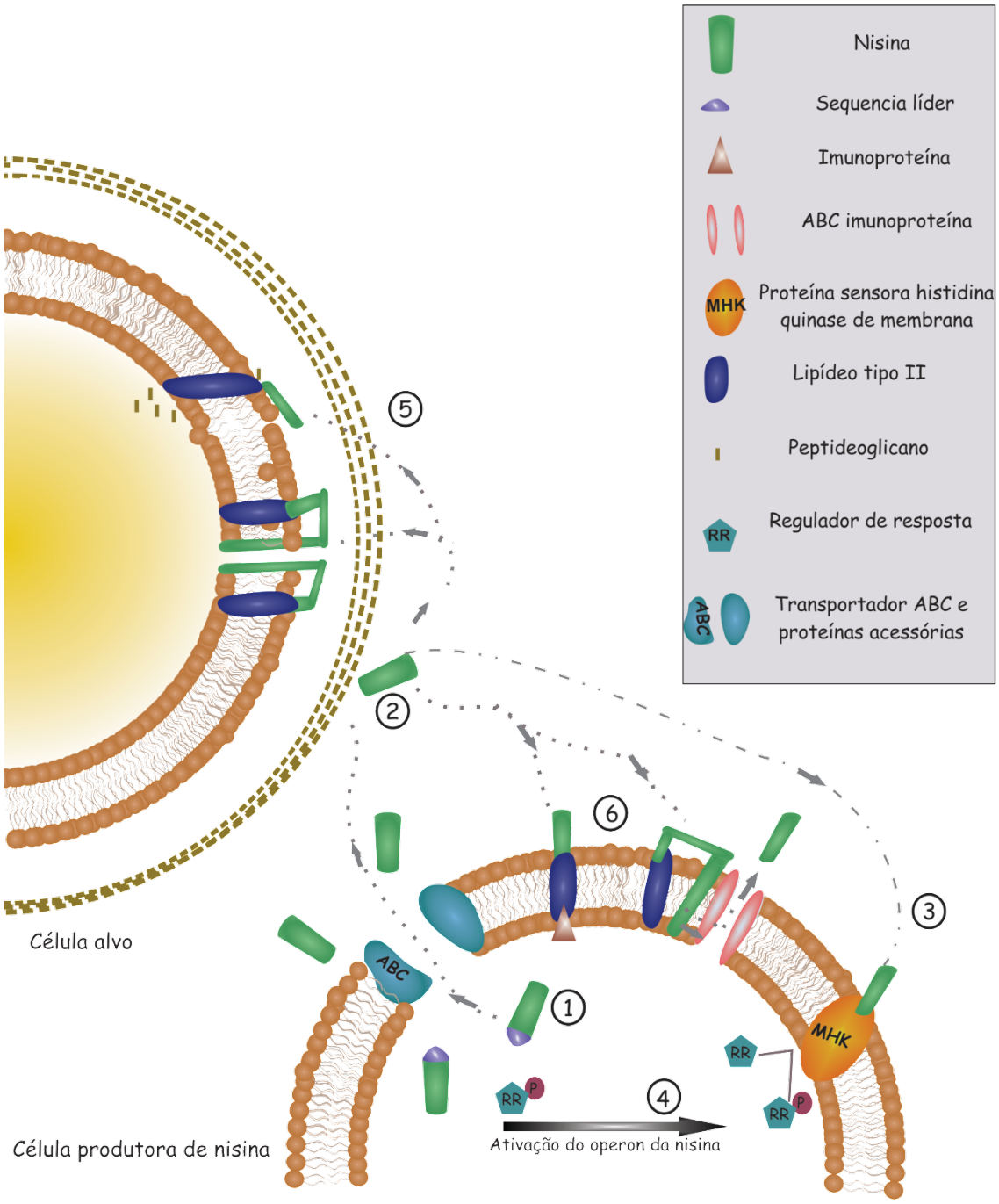


Figura 4. Biossíntese, regulação, autoimunidade e modo de ação da nisina (classe I).

Fonte: Adaptado de Porto *et al*., 2017.

Os genes envolvidos na síntese das pré-bacteriocinas, processamento, transporte, imunidade e regulação estão agrupados em um ou dois "*clusters*" presentes no DNA cromossonal ou plasmidial da bactéria produtora (Nishie *et al*., 2012). Na maioria dos casos a regulação da expressão destes genes é

realizada via transdução de sinal de dois componentes (proteína sensora de membrana histidina quinase (MHK) e regulador de resposta (RR) ou por três componentes (MHK, RR e fator de indução (FI)). A diferença entre os dois sistemas de regulação é que o fator de indução que interage com a MHK no primeiro sistema descrito é a própria bacteriocina presente no meio extracelular. Já no sistema de três componentes, o fator de indução se liga ao MHK e desencadeia a resposta (Drider *et al*., 2006; Lubelski *et al*., 2008).

A síntese das bacteriocinas da classe IIa é regulada via transdução de sinal de três componentes. O peptídeo indutor FI é constantemente expresso via ribossomo como pré-peptídeo. Após ser clivado, o FI é secretado por transportadores ABC. Em uma dada concentração no meio extracelular, esta molécula atua como um fator de indução, interagindo com proteínas de membrana histidina quinase presentes em suas próprias células. Esta interação resulta na autofosforilação do resíduo de histidina desta proteína transmembrana presente na região voltada para o citoplasma. Em seguida, há a transferência do grupo fosfato deste resíduo de histidina para o resíduo de aspartato do regulador, que por sua vez, atua como um ativador dos genes envolvidos na síntese, processamento e regulação da bacteriocina desta classe (Drider *et al*., 2006).

No caso das bactérias produtoras de nisina e outros peptídeos antimicrobianos da classe I, a própria bacteriocina na sua forma ativa no meio extracelular interage com o Nisk, MHK nas células produtoras de nisina, e induz a autofosforilação em seu resíduo de histidina, que posteriormente é transferido para o RR chamado NisR. Ao receber o grupo fosfato, este regulador ativa a expressão do operon da nisina (Lubelski *et al*., 2008). Vale ressaltar que há promotores interno dos genes de regulação *nis*K e *nis*R que são constitutivamente expressos pelas células de *L. lactis (*De Ruyter *et al*., 1996).

3.3 Mecanismo de ação

Diferentes mecanismos de ação são propostos para as bacteriocinas, sendo tais mecanismos diretamente dependentes dos fatores relacionados à espécie bacteriana e condições de crescimento, dose de bacteriocina empregada e grau de purificação (Parada *et al.,* 2007). Os alvos das bacteriocinas nas bactérias sensíveis podem variar desde síntese da parede celular (nisina A, NAI107), formação de poros na membrana plasmática (pediocina, plantaricina, nisina A), síntese proteica (colicina, DF13), replicação, transcrição de DNA (microcina, colicina) e formação de septo (garvicina A, lactococina 972) (Cavera *et al*., 2015).

A ação pode promover um efeito letal bactericida, sem lise ou com lise celular, ou ainda, inibir a multiplicação microbiana com efeito bacteriostático (Cintas *et al*., 2001). Em geral, a permeabilização da membrana das células através da formação de poros é o principal mecanismo pelo qual a maioria das bacteriocinas das BAL exerce seu efeito antibacteriano (Ghrairi *et al.,* 2012). Esse mecanismo ocorre com a bacteriocina se ligando aos receptores na membrana celular da bactéria-alvo. Posteriormente, ocorre a inserção das bacteriocinas na membrana causando dissipação da força próton-motriz e agregação de monômeros com modificação do potencial de membrana e no gradiente de concentração de H^+. São estes efeitos que causam a formação de poros na membrana citoplasmática, provocando a saída de compostos ou altera a força próton-motriz necessária para a produção de energia e síntese de proteínas, o que pode acarretar na perda de viabilidade da célula-alvo (Ghrairi *et al.*, 2012).

3.4 Autoimunidade

As bactérias produtoras de bacteriocinas também expressam proteínas que conferem imunidade contra a ação destes peptídeos antimicrobianos na própria célula. Existem dois mecanismos gerais de proteção descritos na literatura: i) imunoproteína; ii) Trasportador ABC específico contendo duas ou três subunidades (Cotter *et al*., 2005). As imunoproteínas da classe IIa possuem de 88-114 resíduos de aminoácidos; estas sequências não apresentam homologia significativa entre si apesar de possuírem mecanismos semelhantes de proteção, sendo por meio da inativação direta da bacteriocina pela interação com a imunoproteína ou como molécula antagonista competindo com o receptor alvo da bacteriocina (Drider *et al*., 2006; Ennanhar *et al*., 2000).

O mecanismo de ação da imunoproteína da pediocina, ainda, não foi totalmente elucidado. Sua região C-terminal interage com a membrana impedindo a ação da bacteriocina de uma forma direta ou indireta (Fimland *et al*., 2005). Diep e colaboradores (2007) demonstraram o mecanismo autoimune das células produtoras de lactococina A da classe IIa. Primeiramente, esta bacteriocina se liga ao receptor de membrana man-PST e, em seguida, a imunoproteína se liga a este complexo e o inativa.

L. lactis produtoras de nisina apresentam dois mecanismos de autoimunidade frente a esta bacteriocina, que atuam de forma sinérgica: i) Lipoproteína NisI ii) Transportador NisFEG (Stein *et al.*, 2003; Lubelski *et al*., 2008). Stein e colaboradores (2003) demonstraram Nis I está localizada na membrana plasmática voltada para o meio externo e ao se ligar à nisina, impede a formação de poros. Já o transportador NisFEG atua na retirada da nisina já inserida na membrana citoplasmática para o meio externo.

3.5 Resistência

Indiscutivelmente, o aparecimento de linhagens bacterianas resistentes às bacteriocinas vem despertanto grande preocupação na indústria de alimentos e farmacêutica. *Listeria monocytogenes* 412 apresenta uma frequência de linhagens resistentes à pediocina, à nisina, ou ás duas bacteriocinas de $1x10^{-5}$, $5x10^{-4}$ e $9x10^{-8}$, respectivamente (Gravesen *et al*., 2002). A redução expressiva no aparecimento de linhagens resistentes, quando duas bacteriocinas diferentes são utilizadas, demonstra a importância de licenciar outras bacteriocinas além da nisina para a indústria.

Geralmente as linhagens resistentes apresentam modificações na parede celular e/ou na membrana plasmática, variando sua espessura, fluidez ou carga elétrica. Linhagens de *L. monocytogenes* Scott A resistentes à nisina apresentam mudanças na composição de fosfolipídios (Ming; Daeschel, 1995) enquanto que as cepas de *L. monocytogenes* F6891 resistentes a este mesmo lantibiótico apresentam mudanças na hidrofobicidade da parede celular (Davies *et al*., 1996).

Está descrito na literatura que há imunidade cruzada entre os produtores de bacteriocinas da classe II (Eijsink *et al*., 2002). A imunoproteína da sakacina P inativa a ação da pediocina PA-1 e o contrário também ocorre; a imunoproteína da pediocina inativa a sakacina P (Fimland *et al*., 2002). Além disso, os produtores de bacteriocinas da classe II usualmente codificam mais de uma imunoproteína não necessariamente relacionada à bacteriocina produzida, sugerindo que há uma proteção entre algumas bactérias produtoras de bacteriocinas do mesmo grupo (Eijsink *et al*., 2002).

3.6 Aplicação de bacteriocinas

Recentemente, as bacteriocinas têm recebido grande atenção devido ao elevado potencial para aplicação na indústria de alimentos, como agentes conservantes naturais. Do mesmo modo, as indústrias farmacêuticas têm dado uma atenção especial às bacteriocinas, já que estas podem tornar-se um potencial candidato na substituição dos antibióticos de modo a tratar, no futuro, agentes patogênicos resistentes aos mesmos (Yang *et al*., 2014). Ademais, os peptídeos antimicrobianos podem ser utilizados na tentativa de reduzir o uso indiscriminado de antibióticos em produtos alimentares para consumo humano e animal (Sabo *et al*., 2014).

De acordo com Nascimento e colaboradores (2008), as bacteriocinas podem estar presentes nos alimentos por pelo menos três diferentes maneiras: em alimentos fermentados podem ser produzidas "*in situ*" pela adição de bactérias láticas bacteriocinogênicas no lugar das tradicionais culturas iniciadoras; pela adição destas culturas como adjuntas; ou pela adição direta de bacteriocinas purificadas ou parcialmente purificadas. A inoculação de BAL em alimentos, como culturas iniciadoras, tornou-se uma alternativa funcional e altamente aplicável nas indústrias para o controle de bactérias patogênicas, visto que as propriedades organolépticas dos alimentos são mantidas.

3.6.1 Alimentos

O uso de bioconservantes naturais ao invés de agentes químicos é uma estratégia importante para aumentar a vida de prateleira de frutas e legumes minimamente processados. Siroli e colaboradores (2016) utilizaram a cepa produtora de nisina *Lactococcus lactis* CBM21 como agente bioconservante em fatias de maçãs, que limitou o crescimento de leveduras por um período de 28 dias, mantendo as qualidades organolépticas. Kallinteri e colaboradores (2013) estudaram a ação bioconservante da nisina A em queijo do tipo "Galotyri" e observaram que a vida de prateleira desse produto foi de 19 dias, enquanto

que sem a adição deste peptídeo o mesmo deteriorou-se em 14 dias de armazenamento.

Os alimentos cárneos constituem consideráveis fontes de microbiota benéfica e desejável no processo fermentativo; desta forma, cepas probióticas, como *Pediococcus* spp, são utilizadas no controle de *Listeria monocytogenes* em alimentos cárneos. A bacteriocina sintetizada por *Pediococcus pentosaceus*, denominada pediocina, apresentou atividade bactericida contra *L. monocytogenes* 54002 por 2 horas em presunto suíno, com redução celular de 8 log UFC/mL para 5 log UFC/mL, sendo que o crescimento desta cepa patogênica foi controlado por 10 dias (Huang *et al*., 2009). Ao contrário, na ausência desta pediocina, houve crescimento bacteriano de $5,5x10^3$ UFC/g para $3,3\times10^7$ UFC/g.

Outra vantagem interessante da pediocina foi a sua capacidade de se manter estável numa ampla faixa de pH (2.0-8.0) e a 121°C por 15 minutos, tornando-a bastante atraente para o controle microbiano em alimentos cárneos biopreservados por tratamento térmico. As propriedades tecnológicas da pediocina são fundamentais para garantir a sua aplicação em alimentos.

Huang e colaboradores (2009) caracterizaram a pediocina 05-10 sintetizada por *Pediococcus pentosaceus* 05-10 isolado de picles chinês fermentado, que apresentou atividade bactericida contra *L. monocytogenes* 54002 por 2 horas no presunto suíno, com redução celular de $4,5 \times 10^8$ UFC/mL para $4,4 \times 10^5$ UFC/mL, e o crescimento desta cepa foi controlado por 10 dias. Ao contrário, na ausência desta pediocina, houve crescimento bacteriano de $5,5 \times 10^3$ UFC/g para $3,3 \times 10^7$ UFC/g.

A plantaricina, também, é uma bacteriocina muito utilizada em alimentos (Sabo *et al*., 2014). Como exemplo, Enan e colaboradores (1996) isolaram este peptídeo antimicrobiano de *Lactobacillus plantarum* UG1, o qual foi capaz de inibir cepas patogênicas, *L. monocytogenes*, *Bacillus cereus*, *Clostridium perfringens* e *Clostridium sporogenes*.

3.6.2 Uso clínico

Embora a principal aplicação de bacteriocinas seja em alimentos, como agente conservante natural, pesquisas têm verificado o seu potencial uso para fins terapêuticos, como por exemplo, no tratamento de dermatite atópica (Takahashi; Gallo, 2017), úlceras do estômago e infecções do cólon em pacientes com deficiências imunológicas (Culligan *et al*., 2009; Verna; Lucak, 2010).

Ademais, pesquisas mostraram a atividade antimicrobiana de bacteriocinas, possuindo grande eficácia no controle de infecções no trato respiratório (Santagati *et al*., 2012; Taheur *et al.,* 2016). Fernández e colaboradores (2008) demonstraram que a nisina, utilizada como substituta de antibióticos foi muito eficiente no tratamento de mastite estafilocócica bovina.

De acordo com Naghmouchi e colaboradores (2012), a combinação de antibióticos com peptídeos antimicrobianos é uma forma de permitir a redução do uso de antibióticos em aplicações médicas, além de auxiliar na redução de bactérias resistentes aos antibióticos. A bacteriocina subtilisina associada com a clindamicina e o metronidazol mostrou-se eficaz no combate à *Gardnerella vaginalis*, a qual é responsável pela vaginose bacteriana.

A eficácia sinérgica entre bacteriocinas e antibióticos foi medida em estudos "*in vitro*" e "*in vivo*". A nisina combinada com antibióticos convencionais auxiliou na permeabilização da membrana de *Salmonella enterica* serovar *Typhimurium* multirresistente (Singh *et al*., 2013).

Amer e colaboradores (2014) observaram que bacteriocinas derivadas de espécies de *Lactobacillus* (*L. acidophilus* P106 e *L. plantarum* P164) foram eficazes no controle de *Giardia lamblia*, tanto nos ensaios "*in vitro*" (ensaios de inibição de crescimento e aderência) como "*in vivo*", através da estimativa da densidade parasitária, exame histopatológico intestinal e análise ultra-estrutural dos trofozoítos.

No que diz respeito à saúde oral, a cepa *Streptococcus salivarius* produtora do lantibiótico salivaricina A tem reduzido o número de bactérias causadoras da halitose (Tagg *et al*., 2006), enquanto que produtos lácteos suplementados com esta bacteriocina têm ajudado na redução de infecção por *Streptococcus pyogenes*, micro-organismo causador da faringite oral (Dierksen *et al*. 2007). Já na área da saúde sexual e contraceptiva, a lacticina 3147 foi capaz de eliminar espermatozóides de várias espécies animais (Reddy *et al*., 2004; Silkin *et al.,* 2008).

As bacteriocinas representam um sistema de defesa dos micro-organismos, que deve ser amplamente estudado, pois o mecanismo de ação destas biomoléculas ainda não é bem compreendido. Com o surgimento de bactérias multirresistentes, a aplicação de bacteriocinas, como alternativa aos antibióticos disponíveis, vem crescendo significativamente.

4. PRODUÇÃO DE BACTERIOCINA POR *Bifidobacterium* spp.

As bifidobactérias foram primeiramente isoladas e descritas por Tissier (1900). Estes micro-organismos possuem níveis de guanina e citosina elevados, são Gram-positivos, não formadores de esporos, sem motilidade, catalase-negativos e anaeróbicos (Ishibashi *et al*., 1997; Ballongue, 2004; Gomes; Malcata, 1999), sendo algumas espécies aerotolerantes, como no caso do *Bifidobacterium animalis* subsp. *lactis* (Li Q *et al*., 2010). Segundo Ishibashi e colaboradores (1997), existem diferenças morfológicas, havendo o formato de bastonetes curvados, curtos ou bifurcados.

Inicialmente, estas espécies foram classificadas como *Bacillus bifidus communis* e posteriormente, renomeados como *Lactobacillus bifidus*. De Vries e Stouthamer (1967) sugeriram que estes micro-organismos deveriam ser reclassificados como um gênero distinto (*Bifidobacterium*) devido à presença de frutose-6-fosfato fosfocetolase (F6PPK) e ausência de glicose-6-fosfato desidrogenase e aldolase, que são enzimas presentes nas espécies de lactobacilos (Ishibashi *et al*., 1997; Ballongue, 2004; Cheikhyoussef *et al*., 2008).

Nos últimos anos, mais de 34 espécies foram atribuídas ao gênero *Bifidobacterium* (Ventura *et al*., 2007). As bifidobactérias têm sido isoladas de várias fontes, entre as quais a microbiota intestinal de seres humanos, sendo encontradas cerca de 3-7% na microbiota dos adultos e 91% na microbiota de recém-nascidos (Ballongue, 2004; Cheikhyoussef *et al*., 2009).

Algumas espécies de *Bifidobacterium* podem ser consideradas probióticas. De acordo com a FAO/WHO, probióticos são micro-organismos vivos que, quando administrados em quantidades adequadas, conferem benefícios à saúde do hospedeiro (FAO/WHO, 2001). Para isso, tem-se sido sugerido que uma das desejáveis propriedades das cepas probióticas é a habilidade de produzir substâncias, como bacteriocinas, as quais podem

propiciar uma vantagem na colonização e na competição do trato gastrointestinal (Tamime, 2005).

Algumas peculiaridades probióticas têm sido atribuídas às bifidobactérias como: a) indução da produção de imunoglobulina, b) melhoria do valor nutricional dos alimentos através dos substratos não metabolizados pelo hospedeiro, c) atividade anti-carcinogênica e d) síntese do ácido fólico (Gomes; Malcata, 1999; Bevilacqua *et al*., 2003; Touré *et al*., 2003; Collado *et al*., 2005; Cheikhyoussef *et al.*, 2009).

Diferentemente das espécies de *Lactobacillus*, somente um número limitado de estudos tem sido realizado no que diz respeito à produção de compostos antimicrobianos ou de bacteriocinas por cepas de bifidobactérias. Nesse contexto, nosso grupo de pesquisa publicou um trabalho em formato de review (**APÊNDICE 4**), em que reportamos a capacidade das bifidobactérias em produzir bacteriocinas. Além disso, neste mesmo trabalho apresentamos algumas espécies de bifidobactérias, que possuem atividades antagônicas em relação aos micro-organismos patogênicos Gram-positivos e Gram-negativos, devido à produção de bacteriocinas (**QUADRO 4**).

Yildirim e Johnson (1998) isolaram a primeira bacteriocina de *Bifidobacterium*, conhecida como Bifidocin B. Esta bacteriocina mostrou-se muito eficaz contra diferentes micro-organismos patogênicos (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 (Touré *et al*., 2003).

Quadro 4. Bacteriocinas de *Bifidobacterium* spp. e suas principais características.

Bacteriocina	Micro-organismo	Massa molar (kDa)	Estabilidade térmica	Estabilidade frente ao pH	Fase de produção	Produção ótima	Espectro de inibição	Referência
Bifidin	*B. bifidum* NCDC1452	(-)	(100°C-30 min)	4.8-5.5	Após 48h	pH: 4.8	Bactérias Gram-positivas e Gram-negativas	Anand et al., 1984, 1985
Bifidocin B	*B. bifidum* NCFB 1454	3.3	(121°C-15min)	2-12	(12–18 h)	37°C, pH 5.0- 6.0	*Bacillus cereus, Enterococcus faecalis, Listeria monocytogenes, Pediococcus acidolactici, Streptococcus faecalis*, etc.	Yildirim & Johnson, 1998; Yildirim et al., 1999
Bifilong	*B. longum*	120	(100°C-30 min)	2.5-5.0	(-)	(-)	Bactérias Gram-positivas e Gram-negativas	Kang et al., 1989
Bifilact Bb-46	*B. longum* Bb-46	25-127	(121°C-15min)	4-7	(-)	(-)	*Staphylococcus aureus, Salmonella typhimurium, Bacillus cereus, E. coli*	Saleh & El-Sayed, 2004
Bifilact Bb-12	*B. lactis* Bb-12	25-89	Instável em altas temperaturas	4-7	(-)	(-)	*Staphylococcus aureus, Salmonella typhimurium, Bacillus cereus, E. coli*	Saleh & El-Sayed, 2004
Thermophilicin B67	*B. thermophilum* RBL67	5-6	(100°C-5min)	2-10	24h	pH 6 e 40 °C	*Listeria* spp., *Lactobacillus acidophilus*	von Ah, 2006
Bifidin I	*B. infantis* BCRC 14602	3	(121°C-15min)	4-10	18 h	(-)	*LAB* strains, *Staphylococcus, Bacillus, Streptococcus, Salmonella, Shigella, E. coli.*	Cheikhyoussef et al., 2009, 2010
Lantibiotic (Bisin)	*B. longum* DJO10A	(-)	(-)	(-)	1-8h	Auto indução por lantibiótico	*Streptococcus thermophilus* ST403, *Clostridium perfringens, Staphylococcus epidermidis, Bacillus subtilis, Serratia marcescens, E. coli* DH5a.	Lee et al., 2011

(-): Não avaliado.

Fonte: Adaptado de Martinez *et al.*, 2013b.

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

A maior parte das pesquisas tem sido focada na produção de bacteriocinas por BAL, especialmente nos gêneros *Lactobacillus* e *Pediococcus* (Deegan *et al*., 2006), embora as bacteriocinas produzidas por *Bifidobacterium* spp. venham ganhando atenção da indústria de alimentos, já que algumas espécies são reconhecidas como seguras (Picard *et al*., 2005). No entanto, apenas alguns estudos têm sido feitos sobre a otimização da produção de bacteriocinas por *Bifidobacterium* spp., e poucos artigos de revisão foram publicados neste tema (Cheikhyoussef *et al*., 2008; Martinez *et al*., 2013b). No entanto, estes trabalhos concentraram-se principalmente nos processos de separação e purificação, ou na influência do pH e da temperatura na estabilidade de bacteriocinas produzidas por bifidobactérias, em vez de se estudar a otimização da composição do meio de cultivo para *Bifidobacterium* spp. (Yildirim; Johnson, 1998, Yildirim *et al*., 1999, Von Ah, 2006, Cheikhyoussef *et al*., 2010, Lee *et al*., 2011). Tem-se como questões significativas, que devem ser consideradas na produção de bacteriocinas, o alto custo de meios de culturas complexos, normalmente usados em processos de fermentação em larga escala, como *Man, Rogosa e Sharpe* (MRS) ou *Bifidus Seletive Medium* (BSM), bem como a baixa reprodutibilidade e sensibilidade do método de difusão em ágar para a determinação da atividade antimicrobiana (Pongtharangkul; Demirci, 2004). Nesse contexto, o nosso grupo de pesquisa (**APÊNDICE 5** e **APÊNDICE 6**) estudou a produção de BLIS por *Bifidobacterium animalis* subsp. *lactis* utilizando meios de cultivos alternativos de baixo custo, como soro de leite e leite desnatado suplementados com diferentes aditivos (extrato de levedura, inulina e Tween 80).

No trabalho de Martinez e colaboradores (2015), observamos que o uso do leite desnatado (SM) como meio de fermentação proporcionou melhores resultados, em termos de parâmetros de crescimento (μ=0,32 h^{-1} e t_g=2,16 h), quando comparado com o uso do meio de cultivo MRS (μ=0,26 h^{-1} e t_g=2,63 h) (**TABELA 1**).

Tabela 1. Principais parâmetros de crescimento de *B. lactis* BL 04 em meio MRS e leite desnatado (SM) sem (controle) e com suplementação de aditivos (extrato de levedura, Tween 80 ou inulina).*

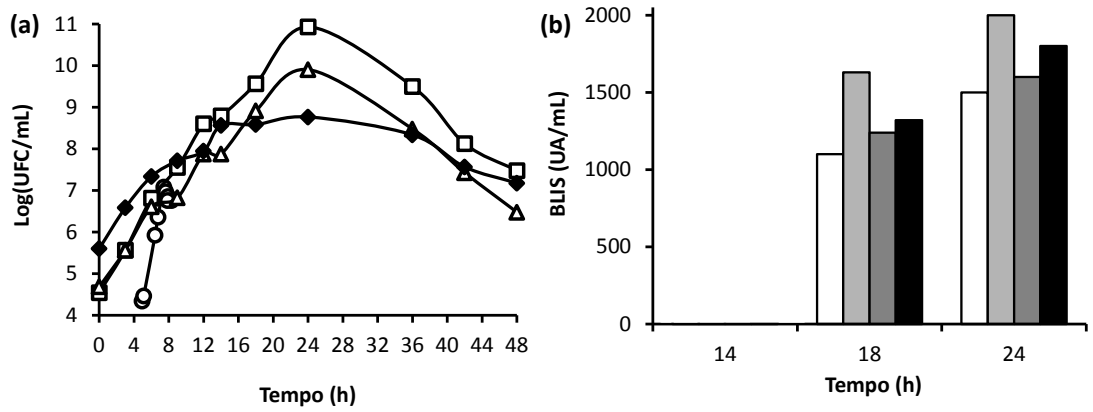
Meio de cultivo	**μ (h^{-1})**	**t_g (h)**
MRS		
Controle	0,26±0,02[a]	2,63±0,02[h]
Extrato de levedura	0,46±0,01[f]	1,49±0,01[c]
Inulina	0,40±0,01[d]	1,73±0,01[e]
Tween 80	0,35±0,01[c]	1,96±0,02[f]
Leite desnatado		
Controle	0,32±0.01[b]	2,16±0,01[g]
Extrato de levedura	0,60±0.02[h]	1,15±0,02[a]
Inulina	0,49±0.01[g]	1,41±0,02[b]
Tween 80	0,44±0.01[e]	1,56±0,01[d]

* Diferentes letras na mesma coluna significam que há diferença estatística entre os valores de acordo com o teste de Tukey (P < 0,05).

Fonte: Martinez *et al.,* 2015.

Provavelmente, a presença de componentes, presentes no leite desnatado, como k-caseína, caseína macropeptídeo, N-acetilglicosamina e ácido N-acetilneuramínico favoreceram o crescimento de *B. lactis* BL04. Além disso, a adição de 1% de extrato de levedura (YE) ao SM favoreceu uma maior produção de BLIS (2000 AU/ml) (**FIGURA 5**) bem como maior biomassa de *B.*

lactis, alcançando na fase exponencial de 24 h uma contagem de 10,9 Log (UFC/mL), μ=0,60 h^{-1} e tg=1,15 h (**TABELA 1**).



Controle (○); Extrato de levedura (□); inulina (△); Tween 80 (♦). (a) Biomassa (Log UFC/mL); (b) BLIS (UA/mL): cores □ (controle), ■ (Extrato de levedura), ■ (inulina), ■ (Tween 80).

Figura 5. Cultivos de *B. lactis* BL 04 conduzidos em shaker a 37 °C e 50 rpm utilizando leite desnatado suplementado com diferentes aditivos.

Fonte: Martinez *et al.,* 2015.

O soro de leite também apresentou-se como meio de cultivo promissor tanto para o crescimento de *B. lactis* quanto para a produção de BLIS, tendo-se obtido um valor de 800 UA/mL, no cultivo em biorreator de bancada (**TABELA 2**) (Balciunas *et al*., 2015).

Tabela 2. Cultivos de *B. lactis* BL 04 conduzidos em shaker e em fermentador de bancada utilizando soro de leite suplementado com diferentes aditivos na concentração de 1% (p/p).

Meio de cultivo	Atividade do BLIS (UA/mL)*	μ_{max} (h^{-1})**
Shaker		
Extrato de levedura	400	0,60 ± 0.1[c]
L-Cisteína	100	0,55 ± 0.1[a]
Tween 80	100	0,53 ± 0.1[a]
Inulina	200	0,60 ± 0.2[bc]
Controle	100	0,53 ± 0.1[a]
Fermentador		
Extrato de levedura	800	0,57 ± 0.2[bc]

* Atividade antimicrobiana de BLIS frente a *L. monocytogenes* ATCC 13932.

** Diferentes letras na mesma coluna significam que há diferença estatística entre os valores de acordo com o teste de Tukey ($P < 0,05$).

Fonte: Balciunas *et al.,* 2015.

Os teores de peptídeos e aminoácidos livres, mesmo presentes em baixas concentrações no soro de leite (Gomes *et al*., 1998; Janer *et al*., 2004) são capazes de estimular o metabolismo de *B. lactis* e de outras bactérias láticas (Thamer; Penna 2005); portanto, além dos teores desses componentes, a rápida aceleração do crescimento de *B. lactis* pode ter sido atribuída também à adição do extrato de levedura (μ=0,60 h^{-1}). Segundo Heng e colaboradores (2007), alguns substratos específicos do meio de cultura, como extrato de levedura e glicose, afetam o crescimento celular e concomitantemente a produção de bacteriocinas de bactérias Gram-positivas.

Cheikhyoussef e colaboradores (2010) e Yildirim e colaboradores (1999) obtiveram valores de atividade antimicrobiana de bacteriocinas produzidas por *Bifidobacterium infantis* BCRC 14602 e *Bifidobacterium bifidum* NCFB1454, ambos em sobrenadantes isentos de células (SIC), de 1600 e 3200 AU/mL, respectivamente. É importante salientar que estes investigadores usaram caldo MRS suplementado com cloridrato de L-cisteína a 0,05% (p/v), que é um meio de cultivo muito mais caro do que o soro utilizado no estudo de Balciunas e colaboradores (2015).

5. PRODUÇÃO DE BACTERIOCINA POR *Lactobacillus plantarum*

L. plantarum é uma das espécies mais difundidas do gênero *Lactobacillus* e vem sendo amplamente utilizada em tecnologias associadas aos alimentos (Brinques *et al*., 2010; Sauvageau *et al*., 2012). Este micro-organismo é heterofermentativo facultativo (Siezen; Vlieg, 2011; Bove *et al*., 2012), ácido tolerante e é considerado GRAS (Brinques *et al*., 2010). Por ser uma espécie heterogênea, está intimamente associada com *Lactobacillus pentosus*, *Lactobacillus paraplantarum* e, mais recentemente, com *Lactobacillus fabifermentans* (Parente *et al*., 2010; Siezen; Vlieg, 2011). Esta relação foi identificada quando mais de 99% de seus RNAr apresentaram sequências idênticas, o que sugeriu alta similaridade fenotípica e genotípica entre as espécies (Parente *et al.,* 2010).

Segundo Ricciardi e colaboradores (2012) *L. plantarum* é uma das espécies mais importantes e versáteis do grupo, podendo ser encontrada como parte da microbiota de alimentos ricos em amido e cereais, carnes, produtos lácteos, vegetais, frutas e bebidas. Diferentes cepas já foram isoladas de diversos nichos, tais como leite fermentado, queijos, pepino fermentado, azeitonas fermentadas, massas, abacaxi, suco de toranja, cerveja de sorgo e cevada, melaço, boza e kefir (Todorov *et al*., 2011). Estas cepas têm capacidade comprovada de sobreviver ao trânsito gástrico e podem colonizar o trato intestinal de humanos e outros mamíferos, sendo consideradas membros da microbiota natural desses nichos (Kleerebezem *et al*., 2003; Mathara *et al*., 2008). A capacidade de se adaptar a uma grande diversidade de nichos pode ser atribuída à habilidade de *L. plantarum* em fermentar ampla variedade de açúcares, tais como lactose, glicose, frutose, maltose, sacarose, manose, entre outros (Todorov, 2008; Prins *et al*., 2010; Brinques *et al*., 2010).

Uma grande variedade de bacteriocinas produzidas por diferentes cepas de *L. plantarum* vêm sendo isoladas e descritas na literatura. Neste contexto o nosso grupo de pesquisa fez um levantamento aprofundado no que diz respeito às bacteriocinas produzidas por *L. plantarum*, além de aspectos sobre a

bioprodução destes compostos antimicrobianos (**APÊNDICE 7**). No **Quadro 5** estão alguns exemplos de bacteriocinas produzidas por *L. plantarum* isoladas de diversos produtos fermentados.

Quadro 5. Exemplos de bacteriocinas produzidas por *L. plantarum* isoladas de diversos nichos ecológicos.

Origem	Micro-organismo	Bacteriocina	Patógeno alvo	Referência
Produtos cárneos	*Lactobacillus plantarum* UG1	plantaricina UG1	*Listeria monocytogenes, Bacillus cereus; Clostridium perfringens, Clostridium sporogenes*	Enan *et al*. 1996
	Lactobacillus plantarum 35d	plantaricina 35d	*Staphylococcus aureus, Listeria monocytogenes*, *Aeromonas hydrophila*	Messi *et al.*, 2001
	Lactobacillus plantarum ST202Ch ST216Ch	bac ST202Ch e bac ST216	*Enterococcus faecium, Escherichia coli, Listeria monocytogenes, Pseudomonas* spp*., Staphylococcus aureus.*	Todorov *et al*., 2010
	Lactobacillus plantarum LT154	plantacina 154	*Enterococcus faecalis, Bacillus* sp*., Staphylococcus* sp*. Salmonella typhimurium*	Kanatani & Oshimura, 1994
Peixes	*Lactobacilus plantarum* PMU 33	plantaricina W	*Listeria monocytogenes*, *Bacillus cereus*, *Staphylococcus aureus*, *Enterococcus faecium*, *Enterococcus faecalis*.	Noopakdee *et al.,* 2009
	Lactobacillus plantarum BF001	plantaricina F	*Staphylococus aures, Salmonela typhimurum, Listeria monocytogenes, P. aeruginosa.*	Fricourt, *et al.*, 1994

Frutas e vegetais	*Lactobacillus plantarum* ST28MS and ST16MS	ST28MS and ST16MS	*Lactobacillus sakei, Staphylococcus aureus, Enterococcus faecalis, P. aeruginosa, Escherichia coli, Acinetobacter baumanii*	Todorov & Dicks, 2004a
	Lactobacillus plantarum C11	plantaricina EF, plantaricina JK e plantaricina A	*Lactobacillus* sp., *Pediococcus* sp., *Leuconostoc* sp. e *Streptococcus* sp.	Anderssen *et al*.,1998; Daeschel *et al.,* 1990
	Lactobacillus plantarum LPC010	plantaricina S and plantaricina T	*Propionibacterium* sp., *Clostridium tyrobutyricum, Enterococcus faecalis*	Jiménez-Díaz *et al.* 1993
	Lactobacillus plantarum ST16Pa	bacteriocina ST16Pa	*Enterococcus faecalis, Enteroccus faecium, Listeria monocytogenes, Listeria innocua, Staphylococcus aureus, Streptococcus* sp., *Pseudomonas* sp.	Todorov *et al.,* 2011
Produtos lácteos	*Lactobacillus plantarum* AMA-K	bacteriocina AMA-k	*Enteroccus* spp. *Escherichia coli, Klebsiella pneumoniae Listeria* spp.	Todorov, 2008
	Lactobacillus plantarum WHE92	pediocina AcH	*Listeria monocytogenes*	Ennahar *et al.*, 1996
	Lactobacillus plantarum LB-B1	pediocina LB-B1	*Listeria, Lactobacillus, Streptococcus, Enterococcus, Pediococcus* e *Escherichia coli*	Xie *et al*., 2011
	Lactobacllus plantarum ST8KF	BacST8KF	*Lactobacillus casei, Lactobacillus salivarus, Lactobacillus cuvatus* and *Listeria innocua*	Powell *et al*., 2007

Cereais	*Lactobacillus plantarum* ST13BR	bacteriocina ST13BR	*P. aeruginosa; Enterococcus faecalis; Klebsiella pneumonia* e *Escherichia coli*	Todorov *et al*., 2004b
	Lactobacillus plantarum ST194BZ	ST194BZ(a) and ST194BZ(b)	*Enterococcus faecalis; Escherichia coli; Enterobacter cloacae; P. aeruginosa*	Todorov, 2005a
	Lactobacillus plantarum 423	plantaricina 423	*Bacillus cereus, Clostridium sporogenes, Enterococcus faecalis, Listeria* spp. e *Staphylococcus* spp.	Reenen *et al.*, 1998

Fonte: Adaptado de Sabo *et al.,* 2014.

- **Carnes:**

  Diversas cepas de *L. plantarum* produtoras de bacteriocinas têm sido isoladas de linguiças obtidas de distintos fabricantes em diferentes períodos de amadurecimento (Garriga *et al*., 1993).

  Enan e colaboradores (1996) isolaram uma substância antimicrobiana produzida a partir de *Lactobacillus plantarum* UG1 obtida da linguiça seca. Esta substância foi capaz de inibir outras cepas do gênero *Lactobacillus* e *Lactococcus* e algumas cepas patogênicas, tais como *Listeria monocytogenes, Bacillus cereu, Clostridium perfringens* e *Clostridium sporogenes.* Este composto antimicrobiano foi caracterizado como uma bacteriocina chamada de plantaricina UG1.

  Messi e colaboradores (2001) isolaram 134 cepas de bactérias ácido láticas de linguiças italianas, que foram testadas para obtenção de produtos antimicrobianos (bacteriocinas). Destes compostos 6% mostraram atividade antimicrobiana contra diversos patógenos alimentares, tais como *Staphylococcus aureus, Listeria monocytogenes* e *Aeromonas hydrophila.* A cepa *Lactobacillus plantarum* 35d produziu a bacteriocina com maior atividade antimicrobiana, que foi denominada plantaricina 35d.

  Um estudo de Todorov e colaboradores (2010), caracterizou bacteriocinas produzidas pelas cepas *Lactobacillus plantarum* ST202Ch e ST216Ch,

isoladas de Beloura ou Chouriço, um produto feito à base de carne de porco, tradicional de Portugal. As bacteriocinas denominadas bacST202Ch e bacST216Ch, foram capazes de inibir o crescimento de diversos micro-organismos Gram-positivos e Gram-negativos considerados deteriorantes de produtos cárneos.

Kanatani e Oshimura (1994) reportaram neste estudo a produção da bacteriocina denominada plantacina 154, produzida a partir da cepa de *Lactobacillus plantarum* LT154, isolada de linguiça seca.

- **Peixes:**

  Noonpakdee e colaboradores (2009) isolaram a cepa *Lactobacillus plantarum* PMU 33 a partir som-fak, uma espécie de produto à base de peixe com baixo teor de sal, tipicamente tailandês. A bacteriocina produzida foi purificada e designada como plantaricina W, podendo inibir um grande número de bactérias Gram-positivas consideradas deteriorantes e patógenos alimentares, tais como *Listeria monocytogenes, Bacillus cereus, Enterococcus faecalis* e *Staphylococcus aureus.*

  Fricourt e colaboradores (1994) isolaram a cepa *Lactobacillus plantarum* BF001 a partir da carne de bagre processada e refrigerada. Esta cepa foi capaz de produzir uma substância antimicrobiana designada plantaricin F, ativa contra algumas bactérias do gênero *Lactobacillus, Lactococcus, Listeria, Micrococcus, Leuconostoc, Pediococcus, Staphylococcus Streptococcus, Salmonella* e *Pseudomonas.*

- **Frutas e vegetais:**

  Duas bacteriocinas, ST28MS e ST26MS, produzidas por diferentes cepas de *L. plantarum*, foram isoladas do melaço e parcialmente caracterizadas. Ambas bacteriocinas demonstraram atividade antimicrobiana incomum contra bactérias Gram-negativas, incluindo *Pseudomonas aeruginosa*, *Escherichia coli* e *Acinetobacter baumanii* (Todorov; Dicks, 2004a).

  Lactobacillus plantarum C-11, isolado de pepinos fermentados (Daeshel *et al*., 1990), produzem bacteriocinas tais como plantaricin EF e plantaricin JK (Anderssen *et al*., 1998). Plantaricina A, anteriormente identificada

incorretamente como a bacteriocina responsável pela atividade antimicrobiana de *Lactobacillus plantarum* C11, induz a produção destas duas bacteriocinas citadas anteriormente (Nissen-Meyer *et al*., 1993).

Jiménez-Díaz e colaboradores. (1993) identificaram duas bacteriocinas produzidas por Lactobacillus plantarum LPC010 isoladas de azeitonas verdes fermentadas. As bacteriocinas foram nomeadas plantaricina S, a qual é produzida durante a fase logarítmica de crescimento e plantaricina T, produzida quando o micro-organismo atinge a fase estacionária de crescimento.

Todorov e colaboradores (2011) isolaram a cepa *Lactobacillus plantarum* ST16Pa a partir da papaia (*Carica papaya*). A bacteriocina produzida por esta cepa foi denominada ST16Pa e demonstrou atividade contra diferentes espécies do gênero *Enterobacter, Enterococcus, Lactobacillus, Pseudomonas, Streptococcus* e *Staphylococcus*, além de alguns gêneros de *Listeria* spp.

- **Produtos à base de leite:**

  Todorov (2008) relatou em seu trabalho a produção de bacteriocina AMA-K produzida pela cepa *Lactobacillus plantarum* AMA-K, isolado de amesi, um produto tradicional feito de leite fermentado, o qual é consumido em diferentes regiões do sul da África, incluindo Zimbábue, África do Sul e Lesoto. A bacteriocina AMA-K demonstrou inibir o crescimento de *Enterococcus* spp., *Escherichia coli, Klebsiella pneumoniae* e *Listeria* spp.

  González e colaboradores (1994) relataram a produção de plantaricina C pela cepa *Lactobacillus plantarum* LL441 isolada de queijo Cabrales. A bacteriocina demonstrou ter modo de ação bactericida e, em alguns casos, com lise celular subsequente.

  Ennahar e colaboradores (1996) reportaram a produção da bacteriocina pediocin AcH por *Lactobacillus plantarum* WHE92 isolada do queijo Munster. A bacteriocina pediocina AcH é naturalmente produzida por diferentes cepas de *Pediococcus acidilactici*. Entretanto, este foi o primeiro

caso relatado em que a mesma bacteriocina apareceu, naturalmente, em uma bactéria pertencente a um gênero diferente.

Xie e colaboradores (2011) demonstraram a presença de uma bacteriocina denominada pediocina LB-B1 ativa contra cepas *de Listeria, Lactobacillus, Streptococcus, Enterococcus, Pediococcus* e *Escherichia coli*, produzida por Lactobacillus plantarum LB-B1 isolado de um produto lácteo fermentado tradicional da China denominado Koumiss, feito por famílias nômades e composto principalmente por leite de égua não pasteurizado.

No estudo de Powell e colaboradores (2007), isolou-se a cepa *Lactobacillus plantarum* ST8KF do *Kefir. Kefir* é uma bebida refrescante e naturalmente gaseificada; possui sabor levemente ácido, é espumante e tem consistência cremosa. A cepa de *Lactobacillus plantarum* ST8KF, isolada a partir desse produto, foi capaz de produzir a bacteriocina BacST8KF, que se mostrou ativa contra *Lactobacillus casei*, *Lactobacillus salivarus*, *Lactobacillus cuvatus* e *Listeria innocua.*

- **Cereais:**

  Todorov e colaboradores (2004b), reportaram neste trabalho a produção da bacteriocina ST13BR a partir da cepa *Lactobacillus plantarum* ST13BR isolada da cerveja de cevada, uma bebida tradicional feita através da fermentação de milho, cevada, farinha de soja e açúcar, produzida na África do Sul. A bacteriocina ST13BR mostrou-se efetiva contra *Lactobacillus casei*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Klebsiella pneumoniae* e *Escherichia coli*.

  Todorov e Dicks (2005a) isolaram a cepa *Lactobacillus plantarum* ST194BZ a partir da boza, uma bebida fermentada típica de países da região dos Bálcãs. A boza é uma das bebidas mais tradicionais da região citada, produzida pela fermentação de diferentes cereais com utilização de leveduras e bactérias ácido-láticas. A cepa ST194BZ foi capaz de produzir dois tipos de bacteriocinas denominadas ST194BZ(a) e ST194BZ(b), sendo ativa contra uma ampla diversidade de patógenos deteriorantes.

  Van Reenen e colaboradores (1998) isolaram a cepa *Lactobacillus plantarum* 423 a partir da cerveja de sorgo. Tal cepa produziu a

bacteriocina plantaricin 423 capaz de inibir uma ampla diversidade de micro-organismos, tais como *Bacillus cereus*, *Clostridium sporogenes, Enterococcus faecalis, Listeria* spp. e *Staphylococcus* spp.

Diversas pesquisas têm objetivado o aprimoramento do meio de cultura e das condições de crescimento para aumentar a produção de bacteriocinas produzidas por *L. plantarum*. Existem inúmeros trabalhos relatando a produção de bacteriocinas por espécies de *L. plantarum* sendo elas*: L. plantarum* ST194BZ, *L. plantarum* ST13BR, *L. plantarum* ST414BZ, *L. plantarum* ST664BZ, *L. plantarum* ST23LD, *L. plantarum* ST341LD, *L. plantarum* AMA-K, *L. plantarum* ST26MS, *L. plantarum* ST28MS e *L. plantarum* ST32, otimizando os meios de cultura utilizando diferentes suplementações, tais como Tween 80, extrato de levedura, caldo de carne, triptona, vitaminas, maltose entre outros compostos (Todorov; Dicks, 2005a; 2005b; 2006a; 2006b; Todorov *et al*., 2000; 2004b; 2007a; 2007b). Além disso, vários pesquisadores (Verellen *et al*., 1998; Sánchez *et al*., 2002; Powell *et al*., 2007; Brinques *et al*., 2010) relataram aumento de produção de bacteriocinas produzidas por diferentes cepas de *L. plantarum* através de modificações dos componentes do meio de cultivo e modificando condições de crescimento.

Apesar dos diversos estudos conduzidos, ainda se tem pouco conhecimento sobre as condições de crescimento requeridas para uma ótima produção de bacteriocinas por *L. plantarum* e, ainda, não foi estabelecido um processo fermentativo ideal (Todorov *et al.*, 2004b). O que se sabe é que o crescimento celular dos *Lactobacillus* é diretamente influenciado pelas condições de pH, temperatura, composição do meio de cultura, taxa de aeração, entre outros fatores.

Nosso grupo de pesquisa estudou o efeito da inulina no crescimento de *L. plantarum* tanto em cultivo estático como em agitador rotativo (**APÊNDICE 8**). Em particular, a máxima concentração celular (X = 5,9 x 10^8 UFC/mL) foi 54% maior em cultivos agitados que os obtidos em cultivos estacionários,

sendo que a velocidade específica de crescimento também foi significativamente maior nos cultivos utilizando agitador rotativo (μ_{max} = 0,53 h^{-1}) em relação aos cultivos estáticos (0,37 h^{-1}); consequentemente o tempo de geração foi proporcionalmente menor (t_g = 1,29 h *versus* 1,85 h) (**TABELA 3**). Concluímos que a agitação melhora a distribuição de nutrientes, e o aumento do nível de oxigênio no meio de cultivo poderia proporcionar altos rendimentos de ATP por mol de substrato consumido.

Tabela 3. Parâmetros cinéticos dos cultivos de *L. plantarum* sem e com agitação (100 rpm).

	Condições de cultivo			
	Shaker	**Shaker + 1% inulina**	**Estacionária**	**Estacionária + 1% inulina**
Concentração celular (UFC/mL)	5,9 x 10^8	5,3 x 10^8	3,2 x 10^8	3,5 x 10^8
μ_{max} (h^{-1})	0,53	0,48	0,37	0,49
t_g (h)	1,29	1,44	1,85	1,40

Fonte: Sabo *et al.,* 2015.

A adição de inulina não aumentou o crescimento da cepa *L. plantarum* ST16Pa, porém sob condições estacionárias, esse ingrediente prebiótico aumentou a velocidade específica de crescimento de 0,37 h^{-1} a 0,49 h^{-1}, reduzindo, portanto, o t_g de 1,85 h para 1,40 h.

Os sobrenadantes livres de células obtidos dos cultivos estacionários, sem a suplementação de inulina, apresentaram valores de atividade antimicrobiana de 1,600 UA/mL frente aos bio-indicadores *L. monocytogenes* L101 e L711, *L. innocua* 2873, 2711 e 2052, e *L. sakei* ATCC 15521, e de 3,200 UA/mL frente a *L. monocytogenes* L104. Por outro lado, em cultivos agitados houve uma redução da atividade antimicrobiana (1,600 UA/mL) contra este último micro-organismo patogênico (**TABELA 4**).

Tabela 4. Quantificação da atividade antimicrobiana das bacteriocinas frente aos diferentes indicadores.

Bioindicadores	Condições de cultivo			
	Shaker	**Shaker + 1% inulina**	**Estacionária**	**Estacionária + 1% inulina**
	Atividade antimicrobiana (AU/mL)			
L. monocytogenes 104	1600	1600	3200	1600
L. monocytogenes 101	1600	1600	1600	1600
L. monocytogenes 711	1600	1600	1600	1600
L. innocua 2711	-	-	1600	-
L. innocua 2873	-	-	1600	-
L. innocua 2052	-	-	1600	-
L. sakei ATCC 15521	-	-	1600	-

- sem atividade antimicrobiana.

Fonte: Sabo *et al.,* 2015.

Tendo em conta que *L. monocytogenes* é um importante micro-organismo patogênico alimentar, os resultados obtidos neste trabalho foram bastante promissores no que diz respeito à produção e aplicação de um bioconservante alimentar natural em nível industrial.

No trabalho de Balciunas e colaboradores (2015), o soro de leite comprovou ser um meio de cultivo promissor tanto para o crescimento de *B. lactis* como para a produção de BLIS. Os meios alternativos de baixo custo devem ser desenvolvidos para competir eficientemente com os meios sintéticos dispendiosos para aplicações em larga escala (Brinques *et al*., 2010).

Nosso grupo de pesquisa avaliou a produção de extratos antimicrobianos (ácido fenilático e bacteriocina) produzidos por *L. plantarum* CECT – 221**,** utilizando soro de queijo hidrolisado e suplementado com vinhaça (**APÊNDICE 9**). O ácido fenilático (PLA) é um ácido orgânico com propriedades antimicrobianas formado a partir do metabolismo da fenilalanina (Phe), sendo produzido por muitos micro-organismos, especialmente por BAL (Valerio *et al*. 2004; Li X *et al*. 2007;

Prema *et al*. 2008; Mu *et al*. 2009; Zheng *et al*. 2011; Rodríguez *et al*. 2012; Rodríguez-Pazo *et al*. 2013).

Na hidrólise sobre a parte proteica do soro de queijo, as proteínas são fragmentadas em aminoácidos livres e peptídeos de diferentes tamanhos (Sinha *et al*., 2007). Desta forma ocorre um aumento da concentração de fontes de nitrogênio, que são essenciais para o crescimento de micro-organismos, favorecendo o desempenho celular e, consequentemente, a produtividade de biocompostos (Corrêa *et al*., 2014).

A **Tabela 5** mostra os resultados obtidos em biorreator operado em regime contínuo. Em particular, a menor taxa de diluição (0,0057 h^{-1}) favoreceu uma concentração máxima de PLA=0,91± 0,085 mM, porém com uma baixa produtividade global (Q_{PLA}=0,00086 ± 0,0001 g/L·h). Já na maior taxa de diluição (0,0393 h^{-1}), a concentração de PLA foi de 0,65 ± 0,127 mM, porém apresentou o maior valor de produtividade global (Q_{PLA}=0,0042 ± 0,0008 g/L·h) (Rodríguez-Pazo *et al*., 2016).

Tabela 5. Valores obtidos dos cultivos de *L. plantarum* CECT – 221, utilizando soro de queijo hidrolisado suplementado com vinhaça.

	Cultivo em biorreator*		
	0,0057 h^{-1}	**0,0207** h^{-1}	**0,0393** h^{-1}
Tempo (h)	-	-	-
$Phe_{t=0}$ (g/L)	-	-	-
Phe_{final} (g/L)	$0,32 \pm 0,042^{bc}$	$0,37 \pm 0,014^{c}$	$0,36 \pm 0,014^{bc}$
$PLA_{t=0}$ (mM)	-	-	-
PLA_{final} (mM)	$0,91 \pm 0,085^{ab}$	$0,81 \pm 0,026^{b}$	$0,65 \pm 0,127^{b}$
Q_{PLA} (g/L·h)	$0,00086 \pm 0.0001^{c}$	$0,00028 \pm 0,0001^{a}$	$0,0042 \pm 0,0008^{b}$
Q_{Phe} (g/L·h)	$0,0039 \pm 0,0002^{a}$	$0,013 \pm 0,0003^{b}$	$0,025 \pm 0,0006^{d}$
$Y_{PLA/Phe}$ (g/g)	$0,22 \pm 0,007^{c}$	$0,21 \pm 0,002^{c}$	$0,17 \pm 0,036^{c}$

* Biorreator operado em regime contínuo sob diferentes taxas de diluição.
Phe: fenilalanina; PLA: ácido fenilático; Q_{PLA}: produtividade global de PLA; Q_{Phe}: taxa volumétrica do consumo de Phe; $Y_{PLA/Phe}$: taxa de conversão de Phe em PLA, calculado da seguinte forma: PLA_{final}-$PLA_{t=0}$)/($Phe_{t=0}$-Phe_{final}). Diferentes letras na mesma coluna significam que há diferença estatística entre os valores de acordo com o teste de Tukey (P < 0,05).

Fonte: Adaptado de Rodríguez-Pazo *et al.*, 2016.

O sobrenadante livre de células (SLC) utilizado para a determinação da atividade antimicrobiana foi obtido a partir da taxa de diluição 0,0207 h^{-1}. No SLC obteve-se um halo de inibição de 12,03 ± 0,50 mm frente à cepa *Carnobacterium maltaromaticum*. Como controles foram utilizados os seguintes produtos comerciais nas mesmas proporções encontradas no SLC: ácido lático (41 g/L), PLA (0,8 ou 7,5 mM) e nisina (1,25 g/L) (**FIGURA 6**). Utilizando 41 g/L de ácido lático comercial, o halo de inibição foi de 6,60 ± 0,63 mm, o que significa que o efeito deste ácido orgânico comercial foi equivalente a 54,9% do valor obtido com o SLC. Por outro lado, a utilização de 0,81 mM de PLA comercial não teve efeito inibidor. Foi necessário aumentar a quantidade de PLA até 7,5 mM para observar um efeito mínimo de inibição (0,10 ± 0,05 mm). Já com a nisina comercial foi possível obter um halo de 5,65 ± 0,65 mm.

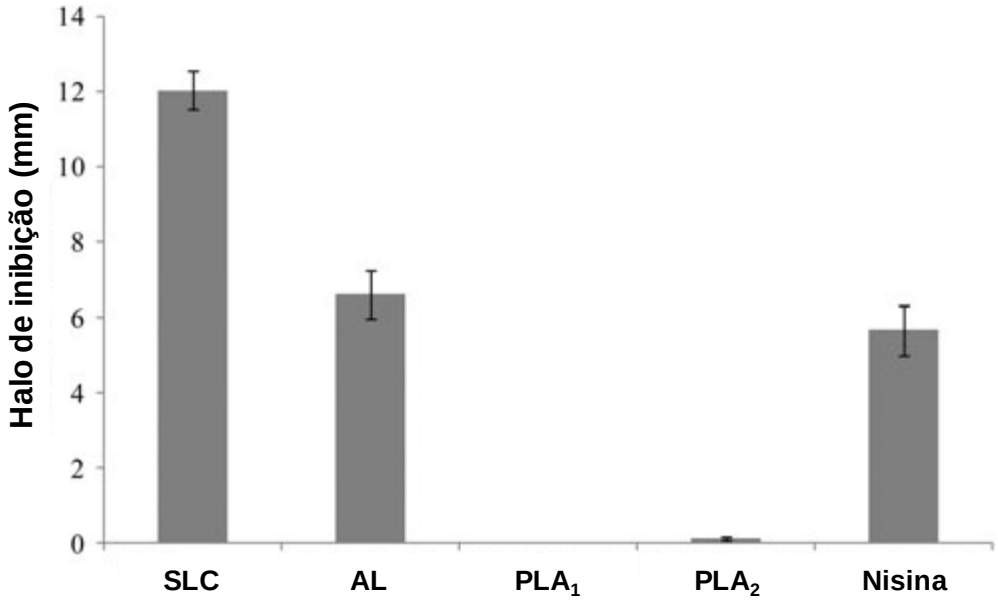


Figura 6. Halos de inibição do sobrenadante livre de células (SLC) de *Lactobacillus plantarum* CECT-221, ácido lático comercial (41 g/L), PLA_1 (0,8 mM), PLA_2 (7,5 mM) ou nisina (1,25 g/L), contra o micro-organismo indicador *Carnobacterium maltaromaticum*. Valores médios (n=3).

Fonte: Rodríguez-Pazo *et al.,* 2016.

6. BIOSSURFACTANTES

Os surfactantes são moléculas anfipáticas constituídas por uma parte hidrofóbica e uma parte hidrofílica (Nitschke; Pastore, 2002). Quando sintetizados por organismos vivos, tais como plantas, micro-organismos, ou o próprio organismo humano, são considerados surfactantes naturais ou biossurfactantes. Entre as vantagens dos biossurfactantes destacam-se: menor concentração dessa biomolécula necessária para reduzir a tensão superficial; maior tolerância às mudanças de pH, temperatura e força iônica, podendo ser usados em uma ampla gama de condições; biodegradabilidade, de modo que não causam problemas ao meio ambiente e toxicidade baixa (Desai; Banat, 1997; Banat *et al*., 2000).

6.1 Biossurfactantes no contexto da biorremediação

A contaminação de águas e solos por hidrocarbonetos aromáticos vem aumentando com o passar dos anos, devido à utilização dessas substâncias em vários segmentos industriais. Hidrocarbonetos são descritos como extremamente poluentes, tóxicos, com potencial carcinogênico e mutagênico para seres humanos. A preocupação com estes compostos aumenta devido às dificuldades em removê-los do meio ambiente.

No contexto da biorremediação, nosso grupo de pesquisa abordou uma série de parâmetros envolvidos na produção e no mecanismo de mobilização e ação de biossurfactante em locais contendo hidrocarbonetos hidrofóbicos relativamente voláteis como alcanos, cicloalcanos, BTEX (benzeno, tolueno, etilbenzeno, xileno), fenol e hidrocarbonetos aromáticos policíclicos, os quais são os grandes responsáveis pela contaminação ambiental (**APÊNDICE 10**).

Muitos hidrocarbonetos aromáticos foram classificados como um grupo prioritário de contaminantes ambientais, que representam uma ameaça à saúde humana e à segurança ecológica devido às suas propriedades tóxicas,

cancerígenas e mutagênicas (Bezza; Chirwa, 2017). Em particular, os hidrocarbonetos aromáticos, tais como o tolueno, são produzidos em grandes quantidades pela indústria petrolífera, e muitas vezes são inadequadamente geridos e despejados ilegalmente, contaminando o solo, as águas subterrâneas, as águas superficiais, a água do mar, a biota e o ar (Farhadian *et al.*, 2010; Lebrero *et al*., 2012). Este hidrocarboneto mono-aromático apresenta uma ampla variedade de usos na indústria, principalmente como componente de gasolina e como solvente para tintas, diluentes, revestimentos, adesivos, tintas, plásticos, óleos, resinas e pesticidas (Farhadian *et al*., 2010; Izquierdo *et al*., 2013).

O tolueno pode ser removido de locais contaminados por processos de biorremediação, através da adição de compostos biosurfactantes, que reduzem a tensão superficial (Souza *et al*., 2014a). Contudo, a aeração e a mistura devem ser otimizadas para assegurar um efetivo coeficiente de transferência de oxigênio (k_La).

O k_La é um dos parâmetros mais importantes para qualquer bioprocesso aeróbico. Este parâmetro é influenciado por uma série de fatores como intensidade de agitação, aeração, tipo e características do biorreator, composição de meios de cultura, e tipo e concentração de micro-organismos (Alves *et al*., 2010; Garcia-Ochoa *et al*., 2010). Por sua vez, a transferência de massa de oxigênio desempenha um papel fundamental no controle do oxigênio disponível para o metabolismo de micro-organismos aeróbios em biorreatores.

Nesse contexto, nosso grupo de pesquisa (**APÊNDICE 11**) estudou o efeito da agitação e aeração em meios com diferentes composições salinas e osmolaridade, tanto na presença como ausência de: tolueno, como um modelo poluente; glicose ou extrato de levedura, como fontes de carbono e nitrogênio, para sua possível utilização em processos aeróbicos de biorremediação. Os resultados deste estudo estão apresentados na **TABELA 6**.

Tabela 6. Valores de k_La (s^{-1}), agitação (rpm), aeração (vvm) e osmolaridade (mOsm/L) em água (W), meio basal salino (BSM), meio de baixa salinidade (LSM), meio Bushnell-Hass (BHM) e meio simulando a composição da água do mar (SW). Amostras suplementadas com 20 g/L de glicose (Glu); 50 g/L de extrato de levedura (YE); 3% (v/v) de tolueno (Tol).

Meio	$k_La \cdot 10^3$ (s^{-1})				
	rpm	50	100	200	300
	vvm				
W	0,5	3,6	4,3	7,6	18,8
	1,0	5,3	6,2	10,0	14,6
	1,5	7,1	9,6	11,7	18,8
BSM	0,5	3,2	3,6	5,0	7,4
	1,0	5,5	5,1	7,8	13,3
	1,5	7,6	7,8	10,1	16,5
BSM + Glu	0,5	4,2	4,4	6,0	10,2
	1,0	6,3	6,0	8,7	14,9
	1,5	8,1	8,3	11,0	17,7
BSM + YE	0,5	2,6	2,7	3,9	6,8
	1,0	2,9	3,6	5,9	12,8
	1,5	4,8	7,7	8,6	11,4
LSM	0,5	4,1	2,9	3,4	8,6
	1,0	6,0	8,0	11,1	18,0
	1,5	7,8	7,4	7,5	16,3
LSM + Glu	0,5	4,5	3,0	6,3	9,8
	1,0	5,5	5,6	9,4	13,9
	1,5	7,9	7,3	8,9	16,6
LSM + YE	0,5	3,4	3,5	3,5	7,2
	1,0	1,6	4,7	6,2	9,1
	1,5	4,6	5,3	7,6	11,0
BHM	0,5	4,1	4,2	8,6	8,0
	1,0	5,8	5,9	11,1	16,0
	1,5	9,9	10,4	13,2	19,7
BHM + Glu	0,5	3,9	4,2	6,4	10,6
	1,0	6,9	6,8	9,2	16,1
	1,5	8,7	9,1	10,7	18,3
BHM + YE	0,5	3,1	3,1	4,1	9,4
	1,0	4,4	4,9	6,3	9,4
	1,5	6,3	7,5	8,5	12,2
SW	0,5	4,9	4,3	6,9	10,1
	1,0	6,4	7,0	9,6	15,5
	1,5	8,5	8,4	11,6	17,7
SW + Glu	0,5	3,3	4,0	6,0	9,3
	1,0	6,0	6,4	9,6	14,9
	1,5	7,9	8,5	12,8	20,7
SW + YE	0,5	3,1	3,7	5,5	9,3
	1,0	4,4	4,9	6,4	12,9
	1,5	5,1	6,0	7,3	14,2
BSM + Tol	0,5	2,6	2,9	5,0	9,8
	1,0	4,9	5,1	6,5	13,0
	1,5	6,6	6,7	8,3	14,7
LSM + Tol	0,5	4,0	2,7	6,0	9,1
	1,0	4,9	3,0	10,6	16,1
	1,5	4,8	7,2	11,5	16,8
BHM + Tol	0,5	1,8	2,2	5,1	10,1
	1,0	5,5	5,1	5,4	15,2
	1,5	5,3	6,9	6,9	17,0
SW + Tol	0,5	2,4	1,2	4,1	7,6
	1,0	2,8	3,7	6,1	9,8
	1,5	4,6	6,7	12,1	15,9

Fonte: Adaptado de Souza *et al.,* 2014b.

Como esperado, as melhores condições foram aquelas que asseguraram os maiores valores de k_La, ou seja, agitação de 300 rpm e aeração de 1,5 vvm. Estes resultados foram devidos à influência da agitação na distribuição e mistura das bolhas de ar, em que uma agitação insuficiente resulta em uma baixa transferência de oxigênio, especialmente em meios altamente viscosos.

Os valores mais baixos de k_La e os mais elevados de osmolaridade foram observados nos meios contendo extrato de levedura, provavelmente, devido à natureza coloidal e viscosidade deste componente, que contribuíram na redução da solubilidade do O_2. Observando a média dos valores, a 300 rpm de agitação e a 1,5 vvm de aeração, o k_La foi de 0,176 s^{-1} e 0,0183 s^{-1} em meio não suplementado e em meio suplementado com glicose, respectivamente. Na presença de extrato de levedura houve um decaimento mais acentuado do valor do coeficiente de transferência de oxigênio (0,0125 s^{-1}). Foi também observada uma redução de k_La (0,0166 s^{-1}) em LSM, BHM e SW na presença de tolueno, provavelmente devido à natureza hidrofóbica deste hidrocarboneto.

De acordo com Badino Jr. e colaboradores (2001), existem algumas equações que correlacionam o k_La com diferentes variáveis operacionais. É bem aceito correlacionar o k_La com a potência volumétrica ($P_g\ V^{-1}$) e com a velocidade superficial do gás (V_s) (Cooper *et al*., 1944), em que a equação pode ser simplificada igualando o termo anterior ao número de potência, resultando na seguinte equação (Garcia-Ochoa; Gomez, 2004; Richards, 1961):

(**Equação 1**)

$$k_La = A(N^3D^2)^{\alpha}[V_S]^{\beta}$$

em que N (s^{-1}) é a velocidade do impelidor, D é o diâmetro do impelidor (m), A é uma constante, e α e β são expoentes empíricos.

Em água destilada, os valores de α (0,132) e β (0,506) da **EQUAÇÃO 1** (**TABELA 7**), que representam as inclinações das retas, que correlacionam o $\ln k_L a$ com $\ln N^3 D^2$ e $\ln V_s$, respectivamente, estão dentro dos valores reportados na literatura para água e soluções líquidas ($0,1 \leq \alpha \leq 0,7$ e $0,3 \leq \beta \leq 0,8$) em diferentes formas de biorreatores e condições operacionais (Özbek; Gayik, 2001; Puthli *et al*., 2005; Marques *et al*., 2009).

Tabela 7. Coeficientes de correlação estimados α e β obtidos a partir da Equação 1. Diferentes letras na mesma coluna significam que há diferença estatística entre os valores de acordo com o teste de Tukey ($P < 0,05$).

Meio	α	β	R^2
W	0,132[a]	0,506[a]	0,995
BSM	0,162[b]	0,606[d]	0,969
BSM + Glu	0,185[d]	0,622[e]	0,979
BSM + YE	0,301[l]	0,654[k]	0,988
BSM + Tol	0,306[m]	0,968[m]	0,979
LSM	0,195[f]	0,869[j]	0,997
LSM + Glu	0,230[i]	0,879[k]	0,981
LSM + YE	0,245[k]	0,892[l]	0,985
LSM + Tol	0,363[o]	0,974[m]	0,989
BHM	0,173[c]	0,510[b]	0,966
BHM + Glu	0,200[g]	0,697[g]	0,998
BHM + YE	0,208[h]	0,771[h]	0,991
BHM + Tol	0,344[n]	1,052[n]	0,987
SW	0,179[d]	0,545[c]	0,968
SW + Glu	0,178[d]	0,802[i]	0,989
SW + YE	0,193[f]	0,877[k]	0,998
SW + Tol	0,237[j]	1,056[n]	0,988

Fonte: Souza *et al.,* 2014b.

Por outro lado, os valores de α e β em BSM, LSM, BHM e SW suplementados com glicose, extrato de levedura ou tolueno foram significativamente maiores que os respectivos valores obtidos na ausência destes compostos, provavelmente, porque sua presença alterou as propriedades físico-químicas da mistura, afetando por consequência a transferência de massa de oxigênio (Marques *et al*., 2009; Farhadian *et al*., 2010; Jamnongwong *et al*., 2010). Tais aumentos foram particularmente elevados na presença de tolueno (α = 0.306, 0.363, 0.344, 0.237 e β = 0.968, 0.974, 1.052 e 1.056, para BSM, LSM, BHM e SW, respectivamente), pois sua hidrofobicidade pode ter promovido a coalescência das bolhas levando ao aumento destas, e consequentemente diminuindo a área interfacial (Özbek; Gayik, 2001; Farhadian *et al*., 2010).

Em continuidade a este trabalho (**APÊNDICE 12**), investigamos a influência do tolueno e da salinidade na produção de biossurfactantes por diferentes micro-organismos (*Bacillus megatherium*, *Bacillus licheniformis* and *Bacillus subtilis*). Nesse trabalho, a cepa *Pseudomonas putida* foi utilizada como comparação, já que é um dos micro-organismos mais utilizados na produção de biossurfactantes. Como resultado deste trabalho, o tolueno inibiu o crescimento de todos os micro-organismos e estimulou a produção de biossurfactante. Nos cultivos em "*shaker*", a cepa *B. subtilis* apresentou o melhor desempenho, sendo capaz de baixar a tensão superficial no meio LSM para 65,5 mN/m na ausência de tolueno, e para 46,5 mN/m no meio BHM adicionado de tolueno, correspondendo a uma redução da tensão superficial de 13,0 e 27,5 mN/m, respectivamente. Em cultivos realizados em biorreator, os melhores resultados foram obtidos no meio LSM, onde *B. subtilis* foi capaz de reduzir a concentração de tolueno de 26,0 para 4,3 g/L em 12 h, reduzindo a tensão superficial para 17,2 mN/m em 18 h. Os resultados deste estudo mostraram que o *B. subtilis* é um interessante produtor de biossurfactante, que poderia ser usado na biorremediação de águas contaminadas com tolueno.

6.2 Biossurfactantes e suas aplicações

Os biossurfactantes apresentam inúmeras aplicações (Banat *et al*., 2000; Kourkoutas; Banat, 2004), destacando-se seu emprego em processos de biorremediação, formulações de produtos de higiene e cosméticos, na indústria alimentícia (emulsificantes), na agricultura e como aditivos para a indústria farmacêutica.

O uso de biossurfactantes na indústria farmacêutica vem se destacando gradativamente (Rodrigues *et al*., 2004; Singh; Cameotra, 2004). A surfactina, um dos melhores biossurfactantes conhecidos, possui várias aplicações médico-farmacêuticas, como a inibição da formação de coágulos, a formação de canais iônicos em membrana, atividade antibacteriana, antifúngica, antiviral e antitumoral. Outras aplicações relevantes dos biossurfactantes incluem o seu papel como agentes antiadesivos de micro-organismos patogênicos, tornando-os úteis para o tratamento de muitas doenças (Gharaei-Fathabad, 2011).

Das e colaboradores (2009) relataram que um biotensoativo produzido por uma cepa marinha, denominada *B. circulans* tinha uma potente atividade antimicrobiana contra patógenos Gram-positivos e Gram-negativos e contra estirpes patogênicas semi-microbianas. Fernandes e colaboradores (2007) investigaram a atividade antimicrobiana de biossurfactantes de *Bacillus subtillis* R14 contra 29 estirpes de bactérias. Os resultados demonstraram que os lipopeptídios têm um amplo espectro de ação, incluindo atividade antimicrobiana contra micro-organismos com perfis multirresistentes a fármacos. Enquanto que Rodrigues e colaboradores (2006a) mencionaram atividade antimicrobiana em MELs (*mannnosylerythritollipids*) produzidos por *Candida antartica*, rhamnolipídeos produzidos por *P. aeruginosa* e lipopeptídeos produzidos por *B. subtilis* 31 e *B. licheniformis*.

Outra aplicação importante dos biossurfactantes consiste na utilização como agentes antiaderentes contra agentes patogênicos para a inibição da adesão destes micro-organismos a superfícies sólidas ou sítios de infecção

(Das *et al*, 2009). A aderência prévia de biossurfactantes às superfícies sólidas pode constituir uma nova e eficaz maneira de combater a colonização por micro-organismos patogênicos (Rivardo *et al*., 2009). Assim, o pré-revestimento de cateteres uretrais de vinil com solução de surfactina resultou numa redução da quantidade de biopelícula formada por *Salmonella typhimurium*, *Salmonella enterica*, *E. coli* e *Proteus mirabilis* (Rodrigues *et al*., 2004a).

A adsorção de biossurfactantes a uma superfície do substrato modifica a sua hidrofobicidade, interferindo nos processos de adesão microbiana e dessorção (Rodrigues *et al*., 2006b) e, neste sentido, a liberação de biossurfactantes por bactérias probióticas *in vivo* pode ser considerada como uma arma de defesa contra outras cepas colonizadoras nas extensões urogenital e gastrointestinal (Van Hoogmoed *et al*., 2004). Assim, foi demonstrado que os biossurfactantes produzidos por *Lactobacillus paracasei* reduzem a adesão de micro-organismos patogênicos e não patogênicos (Gudiña *et al*., 2010a; Gudiña *et al*., 2010b). Rufino e colaboradores (2011) também estudaram o potencial antimicrobiano e antiaderente do biossurfactante Rufisan produzido por *Candida lipolytica* UCP 0988 frente a diferentes micro-organismos da cavidade bucal. Em particular, os autores observaram uma atividade antimicrobiana e anti-adesiva frente ao *Streptococcus mutans* HG985, a uma concentração de 12mg/L de biossurfactante, de 65% e 85%, respectivamente.

A bioincrustação na cavidade oral frequentemente causa graves problemas. A capacidade de *Streptococcus mutans* para sintetizar glucanos extracelulares a partir da sacarose é vital para o início e progressão da cárie dentária. Recentemente, demonstrou-se que alguns compostos biológicos, tais como metabólitos secundários de bactérias probióticas, têm um efeito anti-incrustação. Mais recentemente, Tahmourespour e colaboradores (2011) observaram que os biossurfactantes produzidos por *Lactobacillus fermentum* podem fornecer uma opção para controlar o desenvolvimento de biofilmes e também influenciam a capacidade adesiva de patógenos como *S. mutans*.

A forma mais rápida de saber se um determinado micro-organismo produz biossurfactante, consiste em medir a tensão superficial do meio de cultivo fermentativo (caso o biossurfactante seja extracelular) ou fazer uma extração dos biossurfactantes associados à membrana plasmática com PBS (tampão de fosfato e cloreto de sódio) e medir a tensão superficial. Em geral, uma substância é considerada biossurfactante, quando é capaz de reduzir a tensão superficial do meio em que se encontra em mais de 8 mN/m (Van Der Vegt *et al*., 1991).

Muitos fatores, como aeração, temperatura, pH e a composição do meio de fermentação podem influenciar a produção de diferentes biomoléculas, inclusive de biossurfactantes. Nesse contexto, estudamos os efeitos da temperatura (30, 37 e 40 °C) e o controle do pH na produção de ácido lático, bacteriocinas e biossurfactantes por *Lactococcus lactis* subsp. *lactis* CECT-4434 (**APÊNDICE 13**). Observou-se que a melhor temperatura para produção de biossurfactantes foi 37 °C, apresentando uma redução da tensão superficial de 22,5 e 24,7 mN/m entre 6 e 24 h de cultivo, respectivamente. No que diz respeito à produção de ácido lático, o pH controlado (5,0-5,3) favoreceu o aumento da concentração desse metabólito em 38,8% (30 °C), 40,3% (37 °C) e 47,4% (40 °C) quando comparado aos cultivos sem controle desse parâmetro. Em geral, a produção de bacteriocinas, evidenciada pela formação de zonas de inibição frente às cepas indicadoras *Lactobacillus sakei* e *Staphylococcus aureus*, apresentou maiores valores dentro do intervalo de temperatura 30-37 ºC.

A composição do meio, em relação às fontes de carbono e nitrogênio, tem sido alvo de intensos estudos (Guerra-Santos *et al*., 1986; Desai; Banat, 1997; Cameotra; Makkar, 1998). Neste sentido, Pirog e colaboradores (2004) verificaram a capacidade de *Rhodococcus erythropolis* EK-1 em produzir biossurfactantes tanto sobre substratos hidrofílicos como substratos hidrofóbicos dependendo da composição nutritiva do meio, da natureza das fontes de carbono e de nitrogênio e da duração do tempo de cultivo.

Um dos principais problemas dos biossurfactantes está relacionado com os custos de sua produção. Uma alternativa para melhorar a produção biotecnológica de biossurfactantes em nível industrial pode ser através do emprego de resíduos agroindustriais como soro de leite, águas residuais do moinho de óleos e compostos hemicelulósicos (Moldes *et al*., 2007; Perez-Ameneiro et al., 2015; Gudiña *et al*., 2016).

Assim, Nitschke e colaboradores (2004) estudaram a produção de biossurfactantes mediante o uso de manipueira, melaço, farinha de mandioca, e Nitschke e Pastore (2006) estudaram a produção e propriedades de biossurfactantes sintetizados pela cepa *Bacillus subtilis* LB5a, utilizando água residual de mandioca como substrato. Mahnaz e colaboradores (2004) estudaram a produção de biossurfactantes por *Pseudomonas aeruginosa* MM1011, isolada a partir de óleo cru, sobre 2% de melaço de beterraba, procedente do processo de extração de açúcar, e descobriram que a *Pseudomonas aeruginosa***,** quando cultivada neste meio, produz um rhamnolípidio capaz de reduzir a tensão superficial do meio em até 20 mN/m.

Nosso grupo de pesquisa estudou a produção de biossurfactantes por *Bacillus tequilensis* ZSB10 utilizando brotos de videira como substrato (**APÊNDICE 14**). Neste trabalho, foram aproveitadas simultaneamente as frações hemicelulósicas e celulósica, mediante a formulação de um meio de cultivo salino a partir dos açúcares solubilizados destas frações. Nos cultivos em biorreator, observou-se redução da tensão superficial devido à ação de biossurfactantes (extracelulares e ligados à membrana) (**FIGURA 7**).

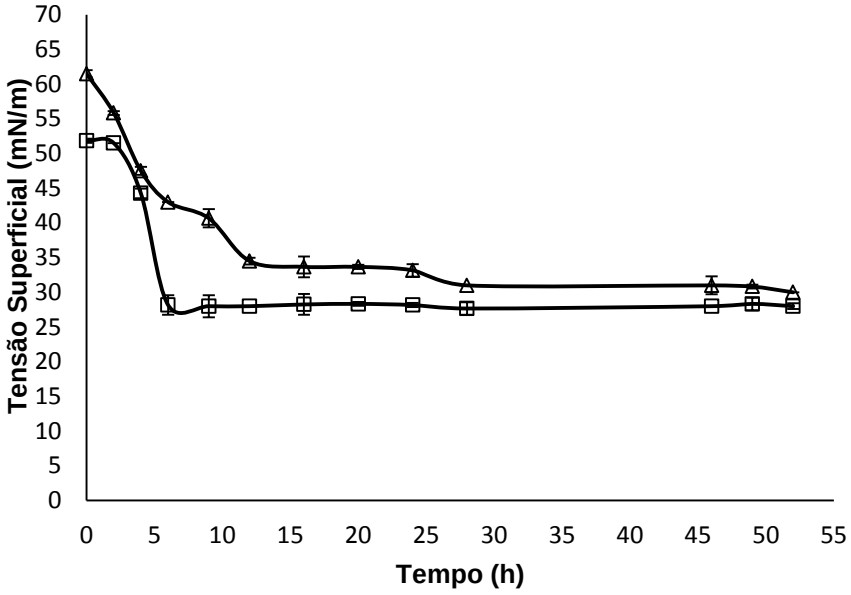


Figura 7. Produção de biossurfactantes por *B. tequilensis* ZSB10 usando meio de cultura salino contend misturas de hidrolisados hemicelulósicos (50 %) e celulósicos (50 %) em biorreator de 2L. Condições de cultivo: 35 °C, 150 rpm, e pH = 7,4. Tensão superficial do biossurfactante extracelular (□) e intracelular (∆).

Fonte: Cortés-Camargo *et al*., 2016.

Notou-se que no meio de cultivo contendo biossurfactantes extracelulares, houve uma maior redução da tensão superficial (TS) (23,7 mN/m) após 6 h de cultivo, sendo que até o final do processo fermentativo (52 h) não houve uma maior redução da TS, indicando que foi alcançada a concentração micelar crítica (CMC). A CMC é definida como a concentração de surfactante necessária para iniciar a formação de micelas. Quando a CMC é atingida, a TS não mais diminui, mesmo adicionando mais surfactante (Shavandi *et al*., 2011). No diz respeito ao biossurfactante ligado à membrana, observou-se que também houve decréscimo da TS (31,5 mN/m) em 52 h de cultivo.

7. CONCLUSÕES E PERSPECTIVAS

Nesse trabalho foram apresentadas a principais contribuições do meu grupo de pesquisa no que se refere à obtenção de produtos de origem microbiana de interesse farmacêutico, alimentar e ambiental.

Com relação à produção de bacteriocinas por *B. lactis* e *L. plantarum*, nossos trabalhos contribuíram para a expansão do conhecimento sobre estes peptídeos antimicrobianos. Ademais, estes trabalhos ressaltaram uma inovação científica e tecnológica no que tange à utilização do soro de leite como meio de cultivo, valorizando o conceito da produção de biomoléculas de alto valor agregado aliado ao aspecto ambiental, já que o soro de leite é um subproduto gerado por indústrias de laticínios e considerado um agente poluidor, por conter uma alta quantidade de substâncias orgânicas.

Na continuação desses estudos, atualmente estamos investigando outros resíduos agroindustriais, como o bagaço de uva, vinhaça de uva e farinha de soja, além de fontes alternativas de carbono e nitrogênio para a obtenção de diferentes bacteriocinas, como plantaricina, nisina e pediocina, e biossurfactantes. Em particular, a farinha de soja, quando suplementada ao soro de queijo aumentou consideravelmente a produção de plantarina, lactato o crescimento celular de *L. plantarum* ST16Pa, além da produção de ácido lático e plantaricina. No caso do bagaço de uva, este resíduo também se mostrou interessante para a produção de novos produtos lácteos funcionais, sendo que do ponto de vista tecnológico diminuiu o tempo de fermentação e aumentou a viabilidade de bactérias probióticas como *L. acidophilus*.

Além dos estudos supracitados, estamos estudando formas de otimizar o processo de produção de bacteriocinas através de ferramentas de biologia molecular.

Nossa pesquisa também inclui estudos de diferentes métodos de purificação para a obtenção de peptídeos antimicrobianos, visando uma futura aplicação industrial tanto na área de alimentos quanto na área farmacêutica. Como perspectiva de aplicação na área de alimentos, estamos estudando a eficiência bioconservante da plantaricina e pediocina em carnes frescas envasadas. Na área farmacêutica estamos realizando ensaios biológicos para verificar a ação da pediocina, após o processo de purificação, em células do sistema imune.

Atualmente estamos desenvolvendo estudos (teóricos e experimentais) na área de nutrição animal e médico-veterinária, onde estamos realizando um estudo de bioprospecção de bactérias probióticas na produção de aves, suínos e peixes.

No que diz respeito à produção biotecnológica de biossurfactantes, estamos realizando estudos de: caracterização físico-química de biossurfactantes produzidos por *Lactococcus lactis* CECT-4434; estabilidade de biossurfactantes em condições extremas de pH e temperatura; propriedades dos biossurfactantes como aditivos. Ademais, estamos investigando a capacidade antimicrobiana dos biossurfactantes frente a diversos micro-organismos patogênicos, a capacidade dos biossurfactantes para formar emulsões e a inibição da adesão de micro-organismos patogênicos a superfícies sólidas ao utilizar biossurfactantes.

Acredito fortemente que com as linhas de pesquisa que atuo tenho contribuído positivamente para a expansão do conhecimento científico, além de buscar novas tecnologias para uma futura aplicação industrial.

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APÊNDICES

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TRENDS IN FOOD SCIENCE & TECHNOLOGY

Review

Lactic acid properties, applications and production: A review

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

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Introduction

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

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

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

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

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

Physico-chemical properties

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

Production technologies and purification

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

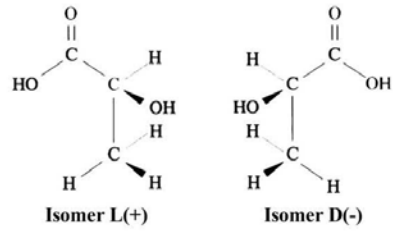

Fig. 1. Structure of D(−) and L(+) isomers of the lactic acid.

Commercial manufacturers

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

Chemical synthesis

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

Fermentation

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

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

Monosaccharides and disaccharides

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

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

Polymeric substrates

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

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

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

Direct fermentation by fungi

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

Fermentation by bacteria

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

Lactic acid purification

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

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

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

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

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

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

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

Fundamentals of biochemistry and metabolism of lactic acid bacteria

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

Lactic acid production from glucose and related fermentation pathways

Homolactic fermentation

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

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

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

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

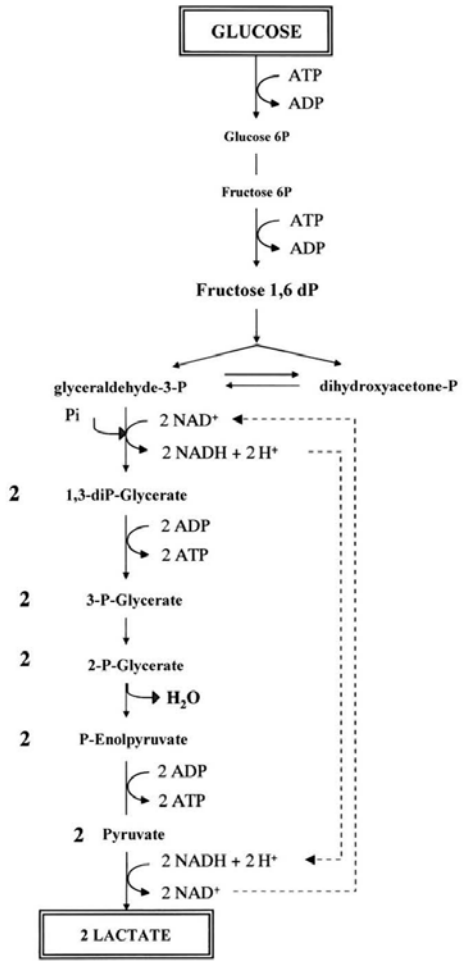

Fig. 2. Scheme of homofermentative pathway of glucose fermentation in lactic acid bacteria. Modified after Axelsson (2004) and Mayo *et al.* (2010).

Heterolactic fermentation

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

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

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

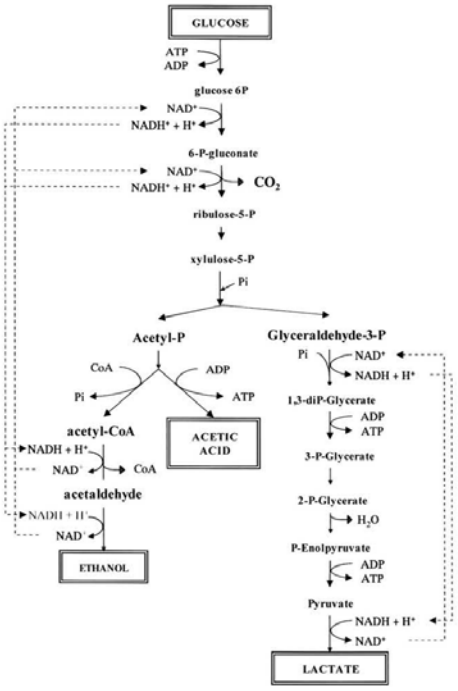

Fig. 3. Scheme of heterofermentative pathway of glucose fermentation in lactic acid bacteria. Modified after Axelsson (2004) and Mayo *et al.* (2010).

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

Lactic acid production from other carbon sources

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

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

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

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

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

Stereospecific lactic acid production

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

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

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

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

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

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

Factors affecting lactic fermentation by bacteria

Nutritional requirements of lactic acid bacteria

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

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

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

(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, & Domí'nguez, 2009).

Acidity

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

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

Substrate inhibition

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

Product inhibition

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

Fermentation technologies

Lactic acid production from sugar solutions

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

Suspended-cell systems

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

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

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

Immobilized-cell systems

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

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

Lactic acid production by simultaneous saccharification and fermentation of polysaccharides

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

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

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

Conclusions

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

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Review

Novel biotechnological applications of bacteriocins: A review

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ABSTRACT

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

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1. Introduction

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

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

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

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

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

2. Classification

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

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

2.1. Class I or lantibiotics

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

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

Table 1
Classification of bacteriocins.

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

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

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

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

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

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

2.2. Class II

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

2.2.1. Subclass IIA

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

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

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

2.2.2. Subclass IIB

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

2.2.3. Subclass IIC

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

2.3. Class III

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

3. Mode of action and structure

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

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

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

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

3.1. Factors affecting bacteriocin efficiency

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

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

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

4. Biotechnological applications

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

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

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

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

4.1. Applications in the food industry

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

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

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

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

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

4.2. Applications in the pharmaceutical industry

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

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

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

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

Table 2
Application of bacteriocins in food biopreservation.

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

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

Table 3
Examples of some bacteriocins and their pharmaceutical applications.

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

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

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

5. Differences between bacteriocins and antibiotics

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

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

6. Resistance to bacteriocins

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

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

7. Biosafety

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

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

8. Purification

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

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

9. Conclusions

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

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Research review paper

Pediococcus spp.: An important genus of lactic acid bacteria and pediocin producers

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ABSTRACT

Probiotics have gained increasing attention due to several health benefits related to the human digestive and immune systems. *Pediococcus* spp. are lactic acid bacteria (LAB) that are widely described as probiotics and characterized as coccus-shaped bacteria (arranged in tetrads), Gram-positive, non-motile, non-spore forming, catalase-negative, and facultative anaerobes. There are many *Pediococcus* strains that produce pediocin, an effective antilisterial bacteriocin. Pediocins are small, cationic molecules consisting of a conserved hydrophilic N-terminal portion containing the YGNGV motif and an amphiphilic or hydrophobic C-terminal variable portion. A number of studies have been developed with *Pediococcus* isolated from multiple biological niches to conduct fermentation processes for pediocin or *Pediococcus* cell production. This review gathers the most significant information about the cultivation, mode of action, and variability of bacteriocins produced by *Pediococcus* spp., emphasizing their applications in the areas of food and clinical practice. This updated panorama assists in delimiting the challenges that still need to be overcome for pediocin use to be approved for human consumption and the food industry.

Contents

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1. Introduction

Lactic acid bacteria (LAB) are commonly used in foods as preservatives and texture, flavor and scent enhancers. These properties result from the ability of these bacteria to produce different types of sugars and metabolites such as lactic acid, acetic acid, ethanol, diacetyl, acetone, exopolysaccharide, specific proteases and bacteriocin by fermentation (Barbosa et al., 2017; Cotter et al., 2013; Gaspar et al., 2013; Gudiña et al., 2015; Mazzoli et al., 2014; Papagianni, 2012; Saad et al., 2013). LAB from the genera *Lactobacillus*, *Bifidobacterium* and *Pediococcus* are commonly found in the mammalian gut microbiota, and some strains are classified as probiotic (WHO/WHO, 2002). The application of this bacterial group in the pharmaceutical and food industries has been increasing given that bacteriocin synthesis often occurs in several LAB strains, resulting in the protection of fermentation products against spoilage and/or pathogenic bacteria.

According to the bacteriocin classification proposed by Cotter et al. (2005), pediocins are biomolecules that can be synthesized by some LAB and present a broad spectrum of antimicrobial activity against Gram-positive bacteria (Papagianni and Anastasiadou, 2009), which highlights its efficient bactericidal effects against pathogenic bacteria, such as *Listeria monocytogenes*. The presence of this microorganism in dairy products, pâtés, sausages and vegetables can be harmful to immunocompromised patients and pregnant women.

Listeriosis is a serious disease that, in spite of its rarity, has a high mortality rate in infected patients (Schuppler and Loessner, 2010). Thus, extensive research on the biosynthesis and antimicrobial effects of pediocins, especially the ones produced from fermented milk cultures, is underway due to increasing industry concern for the control of food-borne pathogens.

Pediocins belong to the bacteriocin group class IIa, characterized as small unmodified peptides (<5 kDa) containing 36 to 48 amino acid residues, a conserved N-terminal portion containing the pediocin box $YGNGVX_1CX_2K/NX_3X_4C$ (X1-4: polar uncharged or charged residues) (Papagianni and Anastasiadou, 2009) and a variable hydrophobic or amphiphilic C-terminal region responsible for cell recognition. It is noteworthy that pediocin has antimicrobial effectiveness even at nanomolar quantities (Papagianni, 2003).

Pediococcus pentosaceus and *Pediococcus acidilactici* are the main species used in i) pediocin production, ii) fermentation processes as a starter (co-culture) for avoiding contamination, and iii) probiotic supplements for animals and humans. As several studies have been published about this topic, this review first focuses on aspects of pediocin covering its biosynthesis, autoimmunity, mode of action, resistance, structural properties and isoforms, with emphasis on molecular data from recent publications. Second, this review addresses biotechnological applications of pediocin and *Pediococcus* spp. in the areas of food and clinical practice. Despite the presence of various beneficial effects of this probiotic described in the literature, there are significant remaining barriers to overcome prior to the use of *Pediococcus* spp. for human consumption.

Nisin is the only bacteriocin approved as a food preservative (Gharsallaoui et al., 2016). The WHO/WHO established all parameters of nisin purity and activity as well as the specific methodology to analyze its antimicrobial activity (WHO/WHO, 1969). The commercial nisin applied in food industry should contain 2.5% w/w of its active form with 10^6 AU/g; the amount of nisin for each type of food (dairy products, meat, bakery wares) was also established by FAO/WHO (FAO/WHO, 2016). Undoubtedly, there is a large avenue for commercial approval of pediocin in the food and pharmaceutical manufacturing sectors: identification of pediocin variant sequences, methods for standardization of antimicrobial activity, and studies of pediocin toxicity or allergenicity. Hence, it is relevant to review pediocin studies and to analyze the state of the art and determine what is needed for its approval as a food preservative or biotherapeutic agent.

2. Pediocin

2.1. Biosynthesis

Bacteriocin production occurs *via* the quorum sensing response, which is related to population density or other environmental stress signaling (Cotter et al., 2005). Generally, these antimicrobial peptides are synthesized as biologically inactive molecules due to the presence of the leader peptide in the N-terminal region. These precursors are transported to the cytoplasmic side of the membrane, where the leader sequence is cleaved and the activated bacteriocin is secreted to the outside of the cell *via* bacteriocin transport or a general secretion system (*Sec*) (Aucher et al., 2005; Cotter et al., 2005; Nes et al., 1996; Xie and Van Der Donk, 2004). For pediocin, the 18- to 24-residue leader peptide is removed after reaching its glycine doublet motif and secreted by the ABC transporter and accessory proteins (Ray et al., 1999). In contrast to other prebacteriocins, the pediocin precursor displays significant biological activity; thus, in order to prevent antimicrobial effects on its own cell, it is suggested that as soon as the pediocin is synthesized, it is quickly transported out of the cell (Ray et al., 1999).

Genes encoding pediocin and proteins involved in pediocin processing, secretion, immunity and expression modulation are arranged in one or two clusters located in a plasmid or in the chromosome (Drider et al., 2006, Kotelkinova and Gelfand, 2002). Pediocin expression is regulated by a three-component system: i) the inducer factor (IF: pheromone or inducer factor), ii) the membrane histidine protein kinase MHK (pheromone receptor) and iii) the response regulator RR. The inducer factor is expressed constitutively at a basal level and exported to the outside of the cell through the ABC transporter system. When a high enough concentration accumulates outside the cell, the inducer factor interacts with the transmembrane histidine kinase, resulting in its autophosphorylation at the conserved histidine residue located in the cytoplasmic side. This phosphate group is transferred to the response regulator, resulting in its activated form (Fig. 1). Thus, this transcription factor binds to the pediocin promoter, triggering expression of bacteriocin-related genes, including the ones responsible for its regulation (IF, RR and MHK). The expression of the inducer factor acts as a positive feedback component in the pediocin circuit, promoting an autoregulated mechanism (Fig. 1). The current literature correlates IF with bacteriocin production and cell growth, although some studies revealed that other environmental signals might have an effect on class II bacteriocin regulation (Cotter et al., 2005). Diep et al. (2000) demonstrated that sakacin A production is temperature-sensitive as well as quorum-sensing-dependent. Under higher temperature conditions (33–35 °C instead of 30 °C) *Lactobacillus sakei* LB706 and *Lactobacillus curvatus* LTH1174 cells reduce IF and bacteriocin synthesis. Interestingly, when an exogenous pheromone is added, cells restore bacteriocin production, evidencing that higher temperature conditions exert an

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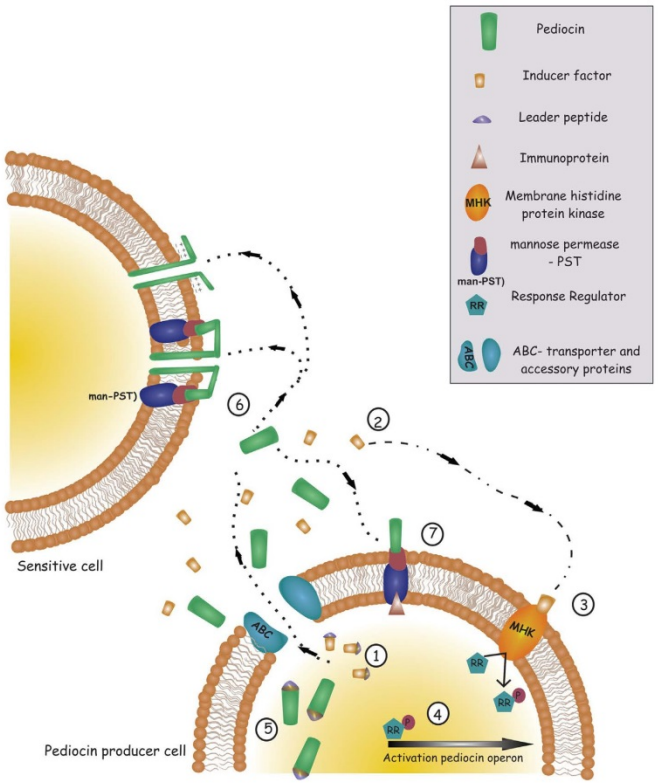

Fig. 1. Pediocin biosynthesis, regulation, autoimmunity and mode of action. Inducer factor (IF) is constantly produced by pediocin producer cells (1), and at a certain IF concentration outside the cell (2), it interacts with MHK (3) present in pediocin producer cell membranes, resulting in MHK autophosphorylation. The phosphate group is transferred to the RR, leading to pediocin operon expression (4). First, the structural pediocin gene is expressed with a leader peptide in its N-terminal portion, which is cleaved by accessory proteins located with the ABC-transporter system (5), followed by pediocin secretion. This bacteriocin binds to sensitive cell membranes through a specific membrane receptor or by electrostatic interaction (6), and water-filled pores are formed, inducing partial or total $\Delta\Psi$ and ΔpH dissipation. Pediocin also interacts with membrane receptors from pediocin producer cells, but immunoprotein presence prevents pediocin pore formation (7).

effect on IF expression. Heterologous expression of the pediocin inducer factor in *P. acidilactici* ATCC25745 cells increase pediocin production by approximately 15-fold in comparison with the corresponding wild-type (Diep et al., 2006). Thereby, it is clear that IF plays an important role in pediocin regulation.

2.2. Autoimmunity

Bacteriocin producers also express immunity proteins protecting themselves from their own antimicrobial peptides. There are two general protection mechanisms presented in the literature: i) a single immunoprotein, and ii) a specialized ABC-transporter system that pumps bacteriocin present at the membrane (Cotter et al., 2005). Class II bacteriocin immunity consists of a single immunoprotein, which varies from 88 to 144 amino acid residues; these sequences do not display significant homology between them in spite of their similar protection mechanisms, which depend on either direct bacteriocin structure inactivation or antagonistic competition with the bacteriocin target receptor (Allison and Klaenhammer, 1999; Eijsink et al., 1998; Ennanhar et al., 2000; Fimland et al., 2005; Quadri et al., 1995). The immunity gene is co-transcribed with the structural bacteriocin gene, as it is generally located in the same operon (Klaenhammer, 1993, Nes et al., 1996).

The pediocin immunity mechanism has not been elucidated yet. Nevertheless, studies on the subgroup of pediocin-like bacteriocins revealed that their C-terminal halves are essential during this process. The immunoprotein hydrophobic region might interact with the membrane in a way that directly or indirectly blocks the action of bacteriocin (Fig. 1). However, it is still unknown whether these immunity proteins bind to bacteriocin or to the receptor (Fimland et al., 2005). Diep et al. (2007) revealed the autoimmune mechanism of lactococcin A, a pediocin-like bacteriocin from class IIa. First, this bacteriocin binds to the man-PST receptor, followed by interaction between an immunoprotein and the complex lactococcin A-man-PST, ultimately resulting in its inactivation.

2.3. Mode of action

Bacteriocins are versatile antimicrobial compounds acting through several mechanisms: a) interference with cell wall formation (nisin,

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nukacinI SK-1, NAI-107); b) disruption of the cytoplasmic membrane (geobacillin I, bac-GM17, plantaricin, dysgalacticin, lactococcin, pediocin-like bacteriocin, mesentericin Y105, lacticin Q, nisin A); c) inhibition of protein synthesis (colicins, cloacin DF13); d) interference with the replication and transcription of DNA (microcin B17, colicins, carocin S2); and e) interference with septum formation (garvicin A, lactococcin 972) (Balciunas et al., 2013, Cavera et al., 2015).

Pediocin, like other Class II bacteriocins, induces partial or total imbalance of transmembrane proton distribution in sensitive cells (Bruno and Montville, 1993; Cleveland et al., 2001; Moll et al., 1999; Papagianni, 2003). This process involves a complex mechanism that is not totally described for pediocins, despite several published studies regarding other bacteriocins from class IIa.

Class IIa bacteriocins possess a putative transmembrane helical domain at their N-terminal region that binds to membrane receptors by electrostatic force (Eijsink et al., 1998, Drider et al., 2006). It has been suggested that the mannose permease-PST receptor is the target of bacteriocin in *L. monocytogenes* cells from the fact that a correlation was verified between this receptor's expression level and bacteriocin sensitivity level in those cells (Gravesen et al., 2002b). Moreover, heterologous expression of mannose permease-PST in *Lactococcus lactis* cells induces sensitivity to class IIa bacteriocins (Ramnath et al., 2004). On the other hand, pediocin PA-1 is able to interact with liposomes from *L. monocytogenes* total lipid extract, and it permeabilizes synthetic phosphatidylcholine vesicles, indicating that no receptor is required (Chen et al., 1997) (Fig. 1). Nevertheless, the experiment was carried out under a higher pediocin concentration in comparison with previous studies (Chen et al., 1997). Fimland et al. (1998) described a peculiarity of pediocin compared with other class IIa bacteriocins, consisting of 15 amino acid residues at its C-terminal region that seem to be essential for targeting cell recognition. This feature might explain the differential target cell specificity and efficiency of pediocin (Fimland et al., 1998).

In a subsequent step, the specific domain in the C-terminus region of class IIa bacteriocin determines its membrane insertion, resulting in the formation of a water-filled pore in a sensitive cell membrane. Chikindas et al. (1993) reported that pediocin PA-1 dissipates the transmembrane electrical potential and acts on salts and amino acid cellular efflux. Purified PD-1 pediocins form pores on the cytoplasmic membrane in *Oenococcus oeni* cells, resulting in K^+ loss. It was also demonstrated that this effect is dependent on low pH conditions, since membrane disruption occurs faster at pH 5.0 than at pH 7.0 (Bauer et al., 2005). The presence of anionic lipids increases the affinity between pediocin PA-1 and phospholipid vesicles (Chen et al., 1997).

2.4. Resistance

Unarguably, the emergence of bacteriocin-resistant strains is a subject of increasing concern in the food industry. It is reported that *L. monocytogenes* 412 cells display a resistance frequency of 1×10^{-5}, 5×10^{-4}, and 9×10^{-8} of spontaneous mutants for pediocin, nisin, and the presence of both bacteriocins, respectively (Gravesen et al., 2002a). A reduction in the expressed resistance demonstrates the importance of different bacteriocins approval for food and pharmaceutical industries.

Generally, resistant cells display cell wall and membrane modifications, such as thickness, electronic charge and fluidity. *Streptococcus pneumoniae* cells exposed to nisin (1 mg/L) result in stable selection for mutant cells, which display a higher MIC for this lantibiotic (from 0.4 to 6.4 mg/L) (Severina et al., 1998).

Cross-immunity occurs among class II bacteriocin producers (Eijsink et al., 2002). The sakacin P immunity protein inactivates pediocin PA-1 and *vice versa* (Fimland et al., 2002). Furthermore, bacteriocin class II producers usually encode several immunoproteins, and some of them are not related to their cognate bacteriocin, suggesting cellular protection against other bacteriocins from this group. It is relevant to mention that no cross-resistance has been reported between class I (nisin) and class II (Metivier et al., 1998; Quadri et al., 1995; Saucier et al., 1995). Nisin-resistant cells of *L. monocytogenes* Scott A present significant changes in phospholipid composition (Ming and Daeschel, 1995). On the other hand, the *L. monocytogenes* F6861 nisin-resistant mutant differs from nisin-sensitive strains only in cell wall hydrophobicity (Davies et al., 1996). Resistance to class II bacteriocins was observed by Robichon et al. (1997). It was verified that *L. monocytogenes* LO28 cells lacking the *rpoN* gene become insensitive to mesentericin Y105. This gene encodes a homologue RNA polymerase subunit σ^{54}, which plays an important role in nitrogen metabolism, pilus production, dicarboxylic acid transport, and toluene and xylene catabolism genes. The absence of these receptors could prevent mesentericin action in sensitive *Listeria* cells.

As previously discussed, expression of mannose permease-PST receptor in sensitive cells is linked to bacteriocin resistance, since this receptor may be the target of pediocin. Recombinant expression of this receptor in non-sensitive cells promotes pediocin antimicrobial activity in *L. lactis* cells (Ramnath et al., 2004), and recombinant cells that lack subunits from man-PST are no longer sensitive to pediocin.

Strains of the food pathogen *Bacillus cereus* that display resistance to alamethicin also have a higher IC50 against pediocin (from 47.8 AU/mL to 58.8 AU/mL) (Meena et al., 2016). It was found that these resistant cells harbor an extra amino-group-containing phospholipid in the membrane that disturbs the interaction of pediocin and alamethicin with sensitive cells (Meena et al., 2016).

2.5. Structural properties

The sequences of pediocin-like bacteriocins present 40–60% amino acid similarity, with a conserved N-terminal hydrophobic region in the YGNGV motif and a variable C-terminal hydrophobic or amphiphilic region (Papagianni, 2003). The substitution of a single amino acid residue results in a significant decrease in antilisterial activity (Sun et al., 2015).

Sequence alignment of class IIa bacteriocins revealed two conserved cysteine residues in the N-terminal region involved in a disulfide bridge, which seems to be essential for supporting the three-stranded antiparallel β-sheet of this region (Drider et al., 2006). The predicted structure of pediocin PA-1 adopts a β-hairpin shape at the YGNGV position when two strands are connected by cysteines (Chen et al., 1997), which confers hydrophilicity on the N-terminal region. Furthermore, pediocins possess a second disulfide bond located in the C-terminal half, providing high temperature stability. Synthetic pediocin PA-1 lacking this second disulfide bond displays structural perturbation in the C-terminal helical domain (Kaur et al., 2004). Johnsen et al. (2000) verified the effect of the methionine residue at position 31 in pediocin PA-1 on the oxidative stress response at 4 °C or room temperature. The replacement of this single residue with alanine, isoleucine or leucine results in the maintenance of pediocin's properties and activity in both temperatures tested, while natural pediocin destabilizes.

2.6. Pediocin variants

Papagianni and Anastasiadou (2009) reviewed different pediocin isoforms, taking into account their temperature and pH stability and proteolytic treatments. Most pediocin isoforms are heat-stable, and most of them remain active at a wide pH range (pH 2.0 to 10.0) (Papagianni and Anastasiadou, 2009). Still pediocin SJ-I (Schved et al., 1993) is unstable at pH > 7 within the range 65–121 °C and pediocin N5p decrease activity at pH < 8 at 4 °C (Strässer de Saad et al., 1995). Interestingly, the main difference among the isoforms is their sensitivity against pepsin, papain, trypsin, chymotrypsin, pronase E and proteinase K. In addition, the different isoforms' inhibitory spectra might be due to differences in amino acid sequence of the primary protein structure, which would result in their distinct antimicrobial activity. Table 1 shows the previously reported properties of pediocin isoforms. Despite the fact that many pediocin sequence isoforms have been identified,

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Table 1
Enzymatic treatment effect on antimicrobial activity of *Pediococcus* spp. CFS (cell- free supernatant) or isolated pediocins. Adapted from Papagianni and Anastasiadou (2009).

Pediococcus strain	Pediocin isoform	Antimicrobial activity		Enzymatic treatment						Reference
		Gram −	Gram +	Pepsin	Papain	Trypsin	α-Chymo trypsin	Pronase E	Proteinase K	
P. acidilactici NRRL B5627	SA-1	−	+	+	+	+	+	ND	−	Anastasiadou et al. (2008b)
P.acidilactici PAC 1.0	PA-1	ND	+	−	−	−	−	ND	ND	Gonzalez and Kunka (1987)
P. acidilactici H	AcH	ND	+	ND	ND	−	ND	ND	ND	Bhunia et al. (1991)
P. parvulus ATO77	ATO77	−	+	−	−	−	−	ND	−	Bennik et al. (1997)
P. damnosus NCFB 1832	PD-1	ND	+	+	+	+	+	ND	−	Green et al. (1997)
P. pentosaceus FBB61	Pediocin A	ND	+	ND	ND	−	ND	−	−	Piva and Headon (1994)
P. pentosaceus ST44AM	ST44AM	±	+	ND	−	−	−	−	−	Todorov and Dicks (2009)
P. pentosaceus ST18	ST18	−	+	ND	ND	ND	ND	−	ND	Todorov and Dicks (2005)
P. pentosaceus S	S	±	+	−	ND	ND	−	−	ND	Yin et al. (2003)
P. pentosaceus L	L	±	+	−	ND	ND	−	−	ND	Yin et al. (2003)
P. pentosaceus KID7	CFS	+	+	ND	ND	ND	ND	ND	ND	Damodharan et al. (2015)
P. pentosaceus ATCC25745	Penocin A	ND	+	ND	ND	ND	ND	ND	ND	Diep et al. (2006)
P. pentosaceus Mees	SM-1	−	+	−	−	−	−	ND	−	Anastasiadou et al. (2008a)
P. pentosaceus K23-2	pK23-2	−	+	−	ND	+	ND	ND	−	Shin et al. (2008)
P. pentosaceus ACCEL	ACCEL	−	+	−	ND	ND	−	−	ND	Wu et al. (2004)

*Antimicrobial spectrum from *Pediococcus* ssp. CFS (cell-free supernatants) or isolated pediocins tested against Gram-positive and Gram-negative bacteria; (+) sensitive, (−) unsensitive or ND: not determined. Antimicrobial activity measured after proteolytic treatment (+) indicates activity following treatment, (−) indicates absence of activity against sensitive cells, ND: not determined.

only a few of them have had their amino acid sequences revealed, making it difficult to discern the causes of variation in their antimicrobial activity. Inactivation upon enzymatic treatment also differs among pediocin isoforms (Table 1). The PD-1, SA-1, and pK23-2 variants maintain their antimicrobial activity even after trypsin incubation, whereas other pediocin isoforms are inactivated.

The common characteristic of all pediocin variants is their efficient antilisterial activity; nevertheless, studies have revealed the specificity of some isoforms. The antimicrobial activity of pediocin S and L from *Pediococcus pentosaceus* S and L, respectively, exhibits an effect over Gram-negative microorganisms (Yin et al., 2003). Both variants inhibited growth of *Klebsiella oxytoca* ATCC 13182, *Proteus vulgaris* ATCC 13315, *Shigella dysenteriae* ATCC 13983, and *Vibrio cholera*. Moreover, only pediocin L shows antimicrobial activity over *Enterobacter aerogenes* ATCC 13048 and *Escherichia coli* ATCC11303. However, neither the S nor the L isoform inhibited growth of Gram-positive microorganisms such as *Streptococcus faecalis* DS-5 or *Staphylococcus aureus* ATCC 25923. Different methodologies applied to evaluate antimicrobial spectrum (agar-well-diffusion assay, spot-on-lawn, LD_{50}), bioindicator species or even the strain used in these experiments may influence the accuracy of the results, leading to an improper comparison of pediocin variants. In the case of pediocin ST44AM, assays have indicated that *Klebsiella pneumoniae* P30 is sensitive to this pediocin, whereas *K. pneumoniae* H2, P31, P39 and XEN 39 are insensitive (Todorov and Dicks, 2009). This also occurs in some Gram-positive microorganisms; *L. lactis* subsp. *lactis* CCRC 12315 and *L. monocytogenes* CCRC 14845 are sensitive to pediocin ACCEL, and no inhibitory effect is observed in *L. lactis* subsp. lactis CCRC 10791 or *L. monocytogenes* RamII. An appropriate standardized method to determine the antimicrobial activity of pediocin should be defined by the FAO/WHO. Indicator strains must be type species or common pathogenic or undesirable bacterial strains, since bacterial susceptibility to bacteriocin varies among strains of the same species as previously discussed.

A global alignment of pediocin sequences was performed in order to verify differences among their isoforms (Fig. 2). All amino acid sequences display the pediocin box $YGNGVX_1CX_2K/NX_3X_4C$ (X1-4: polar uncharged or charged residues). Nevertheless, pediocin produced by *P. pentosaceus* CGMCC 7049, ATCC 2575, harbors several distinct residues, including a serine substituted for the conserved cysteine at 42th position (Fig. 2). It is reported that in addition to the disulfide bond located at the N-terminal end, pediocin possesses a second disulfide bond in the C-terminal half, which is important to provide high temperature stability to this compound. Thus, this amino acid substitution may result in a significant difference regarding tertiary structure and thermal stability between pediocin from *P. pentosaceus* CGMCC 7049, ATCC 2575 and other isoforms. The sequence of pediocin from *P. pentosaceus* IE-3 contig 11 displays a conservative amino acid substitution (valine to leucine) in the pediocin box (Fig. 2). This sequence was obtained from a

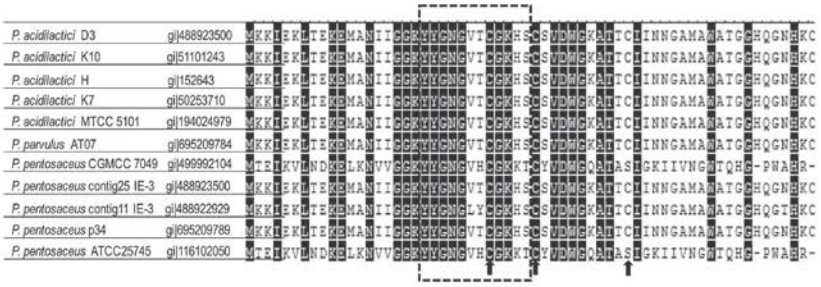

Fig. 2. Pediocin sequence global alignment from *Pediococcus* ssp. generated with ClustalX 2.1. Amino acid residues highlighted by black boxes are conserved in all isoforms including the pediocin box (dashed rectangle) $YGNGVX_1CX_2K/NX_3X_4C$ (X1-4: polar uncharged or charged residues). Black arrows indicate cysteine residues present in all pediocin isoforms except the one located at position 42 that are replaced by serine in *P. pentosaceus* ATCC 25745 and *P. pentosaceus* CGMCC 7049 pediocins.

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draft genome of *P. pentosaceus* IE-3 and so far no biochemical characterization of this bacteriocin has been reported. It is well described in the literature that a single amino acid substitution in the sequence of pediocin (Sun et al., 2015) increases its antimicrobial activity. The identification and biochemical characterization of pediocin isoforms is crucial to select the most effective and appropriate pediocin variant for the industry.

2.7. Pediocin application

Pediocin SA-1 produced by *P. acidilactici* NRRL B-5627 cells is a potential silage additive, as it does not affect the beneficial bacteria *Lactobacillus plantarum*, *Lactobacillus buchneri* and *E. faecium* in maize silages and it inhibits the growth of the pathogenic bacterium *L. monocytogenes* CECT 4032 (Amado et al., 2016). Silage samples with an initial inoculum of 10^5 *Listeria* CFU per gram of maize were ensiled for 30 days. Pediocin SA-1 treatment inhibited *L. monocytogenes* CECT 4032 after 8 days of ensilage, whereas in the control treatment, the presence of CECT 4032 was still detected after 30 days of ensilage (Amado et al., 2016).

ALTA™ 2341 is a fermentation product (by-product) of *P. acidilactici* grown in syrup, vegetable proteins and yeast extract broth is responsible for its flavor, water retention and antimicrobial activity. The purity grade and antimicrobial spectrum of this product are not provided by the manufacturer (Quest International). Schlyter et al. (1993) verified that the antilisterial activity of ALTA™ 2341 and pediocin is similar in different percentage conditions (0%, 1%, 5% and 10%), where 1% of pediocin correspond to 2000 arbitrary units of activity per mL. The presence of ALTA™ 2341 with diacetate in turkey slurries decreases *Listeria monocytogenes* counts after 7 days at 25 °C (2 log10 units) in comparison to a control (Schlyter et al., 1993). ALTA™ 2341 was also tested as a shelf-life extender in cooked chicken breast (Rozum and Maurer, 1997). The presence of 0.75% or 1% of ALTA™ 2341 in chicken samples maintains the aerobic plate counts (APC) in acceptable amounts (APC <1,000,000) for up to 5 weeks of storage at 2 °C (Rozum and Maurer, 1997). The presence of 0.6% of ALTA™ 2341 and acetic acid coagulation in cheese challenged with *L. monocytogenes* (10^6 CFU/g) decrease the colony count by 2 log units when stored at 4 °C for 6 weeks, whereas at 20 °C, pathogen levels increased 0.6 log unit (Glass et al., 1995).

3. *Pediococcus* spp.: an important genus of pediocin-producing bacteria

The identification and characterization of lactic acid bacteria for biotechnological purposes in silage and fermentable ingredients (beans, sauerkraut, cereals, olives and meat derivatives) have gained particular importance in the last few years. The main LAB coccus genera known are *Streptococcus*, *Lactococcus*, *Enterococcus*, *Pediococcus* and *Leuconostoc*. So far, the genus *Pediococcus* spp. is composed of *P. acidilactici*, *P. pentosaceus*, *P. dextrinicus*, *P. damnosus*, *P. parvulus*, *P. inopinatus*, *P. halophilus* and *P. urinaeequi* (Holzapfel et al., 2006; Holzapfel and Wood, 2014).

Regulatory guidelines for probiotic use differ across the globe. Probiotics can be classified as dietary components, drugs, supplements, biological agents (US), functional foods (Japan, China, Malaysia) or biotherapeutic agents (European countries) (Arora and Baldi, 2015). As previously discussed, the FAO/WHO (2002) published a guideline involving phenotypic (*in vitro* and *in vivo* experiments) and genetic analysis to classify a microorganism as a probiotic. No specific regulation for genetically modified (GM) probiotics is established in the European Union, the US or Asia. In these cases, general GMO food legislation is applied. European consumers' perception and current regulations are very restrictive in terms of GMO use. Nevertheless, only microorganisms that have had their material altered by recombinant DNA techniques are considered a GMO. Bacterial modification that uses classical genetic approaches, such as random mutagenesis, is classified as non-GMO. This uncontrolled genetic alteration may affect metabolic processes and could result in toxic products for human health or for the environment. Thus, food safety regulations should also include specific studies of these non-GMOs that have undergone genetic modifications. Indeed, similar to the GRAS status, within Europe, there is the Qualified Presumption of Safety (QPS), a status obtained through rigorous analyses. For each strain of *P. acidilactici* and *P. pentosaceus* that claims QPS status, a scientific report is published by the European Food Safety Authority (EFSA, 2016b). In these studies, identification of the strain is verified along with the absence of antibiotic resistance, and other specific experiments of efficacy and allergenic effects from their use are also conducted. *P. acidilactici* DSM 11673, CNCMI-3237 and *P. pentosaceus* DAS23376, NCIMB 12455 are examples of strains that claim QPS for use as silage additives. *P. parvulus* DSM28875 was just granted QPS status due to the absence of virulence determinants and pathogenicity in human and animals (EFSA, 2016a).

On the other hand, some *Pediococcus* strains have been related to detrimental activity in the food industry as well as in human health. *Pediococcus* spoilage bacteria were identified in an Italian microbrew (Maifreni et al., 2015). Diverse isolated LAB strains show strong ability to form biofilms; among them, two *P. pentosaceus* isolates were identified through 16S RNA sequencing, biochemical and microscopic analysis. Although the use of *Pediococcus* spp. in the food industry, as well as associated with probiotic benefits, their uncontrolled growth in beer fermentation can lead to changes in the final product (flavor, turbidity) (Maifreni et al., 2015). Vancomycin-resistant *Pediococcus pentosaceus* strains were isolated in fuel ethanol fermentation (Murphree et al., 2014). Bacterial contaminants in bioethanol fermentation facilities are considered a serious problem, since bacterial blooms can consume nutrients available for yeast fermentation (Murphree et al., 2014). Septicemia, hepatic abscesses, pneumonitis and bacteremia caused by *Pediococcus* species were reported in patients with chronic underlying conditions or post abdominal surgery and in pregnant women (Barton et al., 2001). Thus, due to the importance of assuring the safety of *Pediococcus* spp., its use as a probiotic requires specific studies that demonstrate a safety assessment of microorganisms throughout the food chain, absence of acquired antibiotic resistance and virulence factors and probiotic viability at the end of shelf life for each strain that claims GRAS or QPS status.

The amount of research involving the *Pediococcus* genus has been continuously growing, especially with *P. acidilactici* and *P. pentosaceus*, characterizing their genetic, molecular and physiological aspects. Both species are commonly used in industry to ferment foods such as vegetables, sausages and meat derivatives due to the preservative properties of pediocin (Papagianni and Anastasiadou, 2009).

Several *Pediococcus pentosaceus* strains were recently identified through genetic (16 s rRNA) and phenotypic analysis (carbohydrate fermentation patterns, acidification kinetics, biofilm formation on microtiter and bacteriocin production assay) (Martino et al., 2013). Ten strains were isolated from pollen grains (Belhadj et al., 2014), six strains from an Indian fermented black lentil dish (Idly) (Vidhyasagar and Jeevaratnam, 2013) and thirty-one strains from cheeses, sausages, fermented milk, powdered milk, sorghum fermented products, bamboo and corn (Martino et al., 2013).

P. pentosaceus cells are spherical bacteria arranged in tetrads. They are Gram-positive, facultative anaerobes, catalase-negative and homofermentative. Hexose fermentation occurs *via* the Embden-Meyerhof-Parnas pathway. The species' 1,83 Mbp of genetic material is composed of 37% guanine and cytosine (GC content), and its transcription results in a total of 5 rRNAs, 55 tRNAs and 1757 mRNAs/proteins (Marakova and Koonin, 2007; Papagianni and Anastasiadou, 2009).

Todorov and Dicks (2009) identified *P. pentosaceus* ST44AM, a pediocin producer strain, in marula fruit (*Sclerocarya birrea*). Jang et al. (2014) isolated the strain *P. pentosaceus* T1 out of fifteen strains obtained from fermented kimchi. Devi and Halami (2011) identified a 4.5 kDa pediocin harboring all properties of the class II bacteriocin group in three species of *Pediococcus*: *P. acidilactici* CB1, *P. pentosaceus* CB4 and

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P. pentosaceus R38. Federici et al. (2014) sequenced the 16S rRNA region from lactic acid bacteria isolates, and nine *Pediococcus* strains present probiotic features such as acid pH and bile resistance and adhesion to Caco-2 intestinal cells (Federici et al., 2014). Oh and Jung (2015) isolated *P. pentosaceus* SW01 and *P. acidilactici* SW05 strains from Omegisool, a Korean fermented corn drink, and found that they displayed 99% 16S rRNA homology to their corresponding species types. Both strains were characterized as Gram-positive, catalase-negative, oxidase-negative bacteria, able to ferment glucose, fructose, mannose and galactose. They survive upon gastric secretion treatment (pH 2.0, 2.5 and 3.0) for 3 h. Moreover, these strains were resistant to bile salts for 24 to 48 h of incubation, and they displayed 65% intestinal cellular adhesion (colorectal adenocarcinoma cell line HT-29).

Carafa et al. (2015) characterized ninety-five LAB isolates from Italian fermentative mountain cheese. 16S rRNA sequences revealed that 26.4% of the isolates belong to the genus *Pediococcus*, and 73.6% to *Lactobacillus* spp. Most *Pediococcus* isolates displayed high similarity to *P. pentosaceus* (16S rRNA gene), and some of them produced conjugated linoleic acid and γ-aminobutyric acid. These isolates could be exploited for probiotic purposes.

Pediococcus halophilus strains are often isolated from miso and soy sauce fermentation since this species is salt tolerant and is able to metabolize citrate and malate, playing an important role during soy fermentation process (Kanbe and Uchida, 1987). *Pediococcus damnosus* NCFB 1832 was isolated from beer and produces pediocin PD-1. As this strain is naturally found during Chardonnay must processing, it could be used as starter (co-culture) to avoid some contaminants due to its pediocin PD production (Nel et al., 2001).

3.1. Culture conditions, pediocin production by Pediococcus spp. and characterization

Recently, several studies demonstrated pediocin production from different *P. pentosaceus* strains, such as *P. pentosaceus* ST44M (Todorov and Dicks, 2009), *P. pentosaceus* CB4 (Devi and Halami, 2011), *P. pentosaceus* R38 (Devi and Halami, 2011), *P. pentosaceus* OZF (Osmanagaoglu et al., 2011), *P. pentosaceus* 3772 (Kingcha et al., 2012) and *P. pentosaceus* NDCD273 (Simha et al., 2012).

A gradual increase of bacteriocin concentration is related to the exponential growth phase from pediocin producers, as it is considered a primary metabolite. The maximum antimicrobial cell-free supernatant (CFS) activity produced by *P. pentosaceus* BCC3772 (Kingcha et al., 2012), *P. pentosaceus* 05–10 (Huang et al., 2009) and *P. pentosaceus* FBB61 (Piva and Headon, 1994) occurs during the transition from the logarithmic phase to the beginning of the stationary phase during the time interval of 12 h–15 h. Higher bacteriocin-like inhibitory substance (BLIS) activity was also observed during the transition from the exponential to the stationary growth phase for *P. pentosaceus* K34 that occurs between 15 and 18 cultivation hours (Abrams et al., 2011). On the other hand, the maximum antilisterial activity was observed during the stationary phase of *P. acidilactici* HA-6111-2 cells (Engelhardt et al., 2015).

Several studies involving *P. acidilactici* strains such as *P. acidilactici* HA6111-2 and *P. acidilactici* HA5692-3 (Albano et al., 2007); *P. acidilactici* UL5 (Dabour et al., 2009); *P. acidilactici* MHC14 (Nieto-Lozano et al., 2010); and *P. acidilactici* J347-29 (Díez et al., 2012) showed their fermentative capability in sausage, milk and dairy raw materials.

Different cultivation parameters, such as pH, dissolved oxygen level and temperature, directly influence BLIS activity. In general, pediocin production occurs satisfactorily from 28 °C to 32 °C, in cultures that are initially neutral, culminating in a final acid environment (pH 4.0) due to acid lactic release (Holzapfel and Wood, 2014). Under semi-aerobic conditions (60% oxygen saturation), *P. pentosaceus* Mees cells produce higher amounts (4-fold) of pediocin SM-1 than in aerobic or anaerobic conditions (Anastasiadou et al., 2008a). *P. acidilactici* NRRL B-5627 cells also produce more pediocin at 60% oxygen saturation than under fully aerobic conditions or in the absence of oxygen. However, the growth (biomass content) and the lactate production reach their optimal level in anaerobiosis (Anastasiadou et al., 2008a). Dissolved O_2 directly affects the oxidative metabolic pathway of *P. acidilactici* NRRL B-5627 cells, leading to an increase in pediocin production, which does not interfere with cell growth. The authors suggested that the pediocin precursor is produced as a primary metabolite; nevertheless, the processing and translocation of the bacteriocin occur at low pH and growth rate (Anastasiadou et al., 2008b).

To maintain high pediocin yields in industrial fermentation, scale-up studies have been carried out in discontinuous batch and fed-batch fermentation. Papagianni and Papamichael (2014) demonstrated that *P. pentosaceus* Mees cells cultivated in stirred tank bioreactors with 60% oxygen saturation displayed higher antimicrobial CFS activity (119%) when 7 g/L·h of sucrose was added during the process. In contrast, 9 or 10 g/L·h of sucrose feeding resulted in BLIS activity decreasing while cell concentration (biomass) remained constant. Nagamouchi et al. (2008) verified antimicrobial CFS activity in *P. acidilactici* UL5 cells cultivated in batches or immobilized in κ-carrageenan/locust bean gum gel beads in a repeated-cycle batch (RCB). The volumetric pediocin PA-1 productivity in RCB is higher (5461 AU/mL·h) in comparison with batch fermentation (342 AU/mL·h) using de Man-Rogosa-Sharpe (MRS) medium. This increment was also observed using whey permeate media; RCB fermentation yielded 6-fold more than batch fermentation. In the RCB process, the pH of 5.5 was controlled by NH_4OH addition and fresh media replacement after 16 h of the experiment, which seems relevant to optimize pediocin production. These findings may facilitate development of large-scale industrial production.

Gradual acidification of *Pediococcus* culture seems to enhance pediocin biosynthesis and processing since bacteriocin transport out of the cell is catalyzed by pH-dependent enzymes. Simha et al. (2012) verified that one pH unit decrease (pH 7.0 to 6.0) led to a 2-fold increase in pediocin PA-1 activity. In *P. pentosaceus* PK34 cells, the highest yield of pediocin bacpK34 occurs at pH ≤ 5.0 (Abrams et al., 2011).

Engelhardt et al. (2015) observed the influence of different pH (3.5, 6.5 and 8.5) and salt presence (7.5% NaCl) conditions on pediocin production in *P. acidilactici* HA6111–2 cells. At pH 3.5, the antilisterial activity decreased (6400 AU/mL) in comparison with cells cultivated at an initial pH of 8.5 or 6.5 (25,600 AU/mL). In the presence of NaCl (7.5%), the growth of *P. acidilactici* HA6111-2 is not inhibited, even though its antilisterial activity reduces considerably.

Carbon source influences pediocin biosynthesis in *P. pentosaceus* Mees cells (Papagianni and Sergelidis, 2015). Biomass production and CFS antimicrobial activity are higher when the bacteria are grown using glucose as the sole carbon source, followed by sucrose and fructose. Using different carbon sources, pediocin SM1 production is a growth-associated product and, hence, a primary metabolite (Papagianni and Sergelidis, 2015).

4. *Pediococcus* spp. application

The application of bacteriocins as biopreservatives has received increasing attention in recent years (Cotter et al., 2013, Deegan et al., 2006, Martinez et al., 2013). *Pediococcus* cultures are marketed today as protective cultures against common food spoilage bacteria and pathogens. Consequently, the application of such cultures may lead to improvements in food quality and sensory attributes by controlling spoilage microbiota. (Bhowmik and Marth, 1990).

4.1. Food

Due to the increased search for functional foods, the addition of probiotics in formulations has become a common worldwide practice. LAB species are the ones commonly selected for this purpose provided that they usually adapt to the gut microbiota and they are acknowledged as safe in this field. According to the FAO/WHO (2002), probiotics are live microorganisms that confer health benefits to the host when

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administered in adequate amounts. The probiotic ability of LAB to promote gut microbiota balance occurs due to their resistance to low pH and bile salts present in the intestinal tract.

The use of pediocin-producing LAB as starter culture in the food industry for *L. monocytogenes* control has been considered a relevant alternative, since the organoleptic proprieties of food products are maintained. Pediocin producers have been isolated from meats, sausages, pickles, and dairy products. In addition to playing an important role during several fermentation processes, these strains are also capable of inhibiting undesirable microorganisms through pediocin production.

Barbosa et al. (2015) evaluated the resistance of *P. acidilactici* HA-6111-2 through simulated gastrointestinal conditions and cell viability after spray-drying procedures. In the presence of pepsin (pH 3.0) or bile salts, this strain exhibits only < 0.05 and 0.2 log unit of reduction, respectively, in the quantity of viable cells, respectively. Furthermore, when dried for 60 days at 4 °C or at room temperature, the cultures displayed 0.6 and 2.9 log unit decreased viability, respectively. Altogether, these results demonstrated that *P. acidilactici* HA-6111-2 is a potential probiotic candidate for the food industry. Federici et al. (2014) identified LAB isolates from Ciauscolo salami and examined their acid resistance, bile tolerance, adhesion to Caco-2 cells and antibiotic resistance. *P. pentosaceus* isolates display resistance to acid pH conditions for 3 h and to a physiological concentration of bile during 4 h. Finally, these strains exhibit a medium degree of adherence (31%) to an *in vitro* intestinal epithelium model, Caco-2 cells.

P. pentosaceus 05-10 was isolated from a traditionally fermented Sichuan pickle and was characterized as a pediocin producer by Huang et al. (2009). This bacteriocin, whose MW is over 6.5 kDa, presents a bactericidal effect against *L. monocytogenes* 54002. Pork hams pretreated with pediocin 05-10 have significantly less *Listeria* growth than samples incubated with inactive bacteriocin when stored for 10 days at 4 °C. Moreover, the pediocin 05-10 isoform is stable across a pH range of 2–10, which makes it a promising candidate for application in meat products since listeriosis outbreaks frequently occur at neutral to basic pH (Paukatong and Kunawasen, 2001).

Originally isolated from Portuguese "alheira" sausage, *P. pentosaceus* K34 produces pediocin bacPPK34 of MW 2.5–6.2 kDa (Abrams et al., 2011). The maximum pediocin expression was obtained at the early stationary growth phase (15–18 h), displaying 12,800 AU/mL against *L. monocytogenes* (Abrams et al., 2011). *P. acidilactici* HA-6111-2 and *P. acidilactici* HA-5692-3 strains were isolated from the same food matrix, and they were observed to produce pediocin of MW 3.5 and 6.5 kDa, respectively. Both bacteriocins repressed cell growth of *E. faecium* HKLSH (3200 AU/mL) and *Listeria innocua* N27 (1600 AU/mL) (Albano et al., 2007, 2009).

4.1.1. Milk

Pediocin production was also evaluated in *P. acidilactici* strain F (PAF) co-fermented with *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. bulgaricus in milk fermentation (Somkuti and Steinberg, 2010). The last two species are commonly used as a starter culture mix due to their capacity to hydrolyze lactose to glucose and galactose. No antilisterial activity is detected when *P. acidilactici* cells are cultivated as monoculture, since this strain is not able to consume lactose as a substrate. On the other hand, co-fermentation of these three strains reached 3200 AU/mL against *L. monocytogenes* Scott after 8 h of growth. No significant difference is observed in antilisterial activity whether skim (0%) or fat (2%) milk is used. Furthermore, inoculation of milk samples with 10^3 CFU/mL of *L. monocytogenes* Scott A, *P. acidilactici* and the other two LAB decreases viable *Listeria* cells (10^2 CFU/mL) in comparison with fermentation in the absence of *P. acidilactici cells* (~10^3 CFU/mL) after 16 days of storage at 4 °C. Nevertheless, more studies regarding the specific ratio of cell concentrations, pediocin production and antilisterial activity in milk should be done in order to achieve complete elimination of *Listeria* cells (Somkuti and Steinberg, 2010).

4.1.2. Sausage and pork meat

Kingcha et al. (2012) observed a significant decrease of *L. monocytogenes* ATCC 19115 growth in Nhan, a Thai traditional fermented pork sausage, when it was inoculated with *P. pentosaceus* BCC 3772 cells. As shown by a spot-on-lawn assay, ATCC 19115 counts decreased 3.2 log CFU/g after BCC3772 (10^6 CFU/g) addition within 24 h of fermentation in comparison to the control (addition of non-pediocin-producing strains). The antimicrobial activity was attributed to production by *P. pentosaceus* BCC3772 of pediocin that shows 100% amino acid identity with the commercial pediocin PA-1 isoform. A correlation was observed between antilisterial activity and *P. pentosaceus* BCC3772 inoculum, since a lowe concentration of this strain (10^4 CFU/g) in Nhan results in a reduction of just over 2 log CFU/g of *Listeria* cells after 24 h followed by a slight increase in *L. monocytogenes* counts (0.4 log CFU/g) after 36 h. In addition, the authors suggested that this strain is a suitable candidate for *Listeria* control in fermented pork sausage, given that no significant changes of Nahn's organoleptic properties were observed (Kingcha et al., 2012).

Dry Spanish fermented sausages were incubated with *L. monocytogenes* CECT4031 and the pediocin-producing strain *P. acidilactici* MCH14 (Nieto-Lozano et al., 2010). After 60 days, *Listeria* counts decreased 2 log cycles in comparison to the control assay (non-bacteriocin producing strain). Antimicrobial activity was measured in bacteriocin units (BU), of which one unit is defined as the amount of bacteriocin that inhibits 50% of bioindicator bacteria growth. Furthermore, addition of purified pediocin PA-1 (5000 BU/mL) to frankfurter sausages results in inhibition of *L. monocytogenes* CECT4031 and *Clostridium perfringens* CECT 376 growth (approximately 2 log cycles) over 60 days of storage 10 °C when compared to their corresponding control samples (Nieto-Lozano et al., 2010).

4.1.3. Fish and seafood

P. acidilactici ALP57 was isolated from non-fermented shellfish (oyster, mussels, clams). This strain synthesized pediocin bacALP57 (MW approximately 6.5 kDa), whose sequence has high similarity to that of pediocin PA-1 (Pinto et al., 2009). The antimicrobial activity of bacALP57 included typical Gram-positive pediocin-sensitive bacteria as well as some strains isolated from seafood. *P. acidilactici* ALP57 produces maximal BLIS activity against *L. monocytogenes* ESB54 (seafood isolate) during its exponential growth phase (12,800 AU/mL). It is known that some *L. monocytogenes* strains are resistant to high salt concentration and low temperatures. More interestingly, listeria outbreaks had previously been reported due to seafood contamination. Thus, the authors emphasized the importance of the identification and characterization of the first pediocin producer strain isolated from seafood, insofar as this isolate is adapted to seafood matrices and shall be interesting to apply as a means of listeriosis control in these products.

Jang et al. (2015) studied the antilisterial activity of *P. pentosaceus* T1 strain in salmon-based media. The minimum inhibitory concentration of this pediocin isoform (20 mg/mL) is lower than that of nisin (over 20 mg/mL) in its bacteriostatic or bacteriocide effect on *L. monocytogenes* cells. Inoculation of salmon fillets with *Pediococcus* culture results in a decrease of *L. monocytogenes* growth from 10^7 CFU/mL to 10^3 CFU/mL.

4.1.4. Bread

Cizeikiene et al. (2013) evaluated the bacteriocin activity in bread storage of *P. pentosaceus* KTU05-9, a strain isolated from Lithuanian rye sourdoughs. In the bakery industry, *Bacillus subtilis* spores are frequently found as a contamination source resulting in ropiness of bread. To verify the antimicrobial activity of this *Pediococcus* strain in wheat bread samples, 5.10^4 spores of *B. subtilis*/g was added to the moisture in the presence or absence (control) of KTU05-9 as a starter.

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The presence of 20% *P. pentosaceus* KTU05-9 delays visual ropiness (6 days) in comparison to the control samples (1.5 day) at 23 °C. It is relevant to mention that the properties of the bread were maintained in this experiment. In addition, anti-mold activity on the bread surface was studied by single-cell suspension spraying of *P. acidilactici* KTU05-7, and *P. pentosaceus* KTU05-8 and KTU05-10. Addition of pure culture (5.10^4 CFU/cm^2) from each previously described strain inhibited fungal growth during 8 days of storage at 15–16 °C wrapped in polythene bags, whereas large amounts of mold were observed in the control bread samples (no inoculation with *Pediococcus* cells).

4.1.5. Wines

In the wine-making process, an uncontrolled ratio of yeast, LAB and acetic acid bacteria (AAB) cells results in spoilage of the final product. Díez et al. (2012) verified the antimicrobial activity on oenological LAB of pediocin PA-1 produced by *P. acidilactici* J347-29 in different conditions of grape must and ethanol concentration. The maximum pediocin activity occurs at 60% grape must and 2% ethanol or 20% grape must and 2–4% ethanol. Purified pediocin PA-1 displays an IC_{50} of 320 ng/mL for several wine LAB and an IC_{50} of 19 ng/mL for *Oenococcus oeni*, a typical oenological LAB. The combination of metabisulfite – a common antimicrobial compound used in the wine industry – and pediocin PA-1 leads to metabisulfite MIC decrease in *O. oeni* cells (2-fold reduction) and in wine LAB cells (4-fold decrease) in the presence of low pediocin PA-1 concentration (10 ng/mL). This study demonstrated that *P. acidilactici* J347-29 is a good candidate for microbiological control in wine making.

4.2. Clinical studies

Studies have demonstrated how the complex interaction of host and probiotic microbiota could impact a wide variety of aspects of the host's health (Alexandre et al., 2014; Canibe and Jensen, 2012). Probiotic research focusing on prophylaxis such as the control of inflammatory diseases, gastrointestinal disturbances, allergies, diarrheas, tumors and pulmonary infections has gained wide attention due to the promising but still preliminary results of *in vitro* and *in vivo* (animal cells and microorganisms) studies. Table 2 summarizes the clinical applications reported for *Pediococcus* spp.

4.2.1. In vitro experiments

Jensen et al. (2012) performed *in vitro* studies aiming to verify cell viability of several probiotic LAB upon simulated gastrointestinal transit, intestinal human cell adhesion and trans-epithelial electrical resistance. The strain *P. pentosaceus* Q3, isolated from rye, displays the same cell viability after exposure to simulated gastric juice (pH 3.0) for 180 min and small intestinal juice, including pancreatin and bile, for 240 min. Moreover, when HT-29 cells and Caco-2 cells are exposed to 10^6 CFU *P. pentosaceus* Q3, this strain presents 6.2% adherence in HT-29 cells and 4% in Caco-2 cells. Mukherjee et al. (2013) revealed a slight decrease of *P. pentosaceus* CRA51 cell viability after simulated gastrointestinal conditions for 120 min (from 6.02 $\log_{10}$ CFU/mL to 5.77 $\log_{10}$ CFU/mL).

P. pentosaceus OZF, originally isolated from human breast milk, was the study subject of Osmanagaoglu et al. (2010). First, it was determined that this strain is resistant to simulated gastrointestinal tract conditions (low pH, bile, pepsin and pancreatin presence) and no γ-hemolysis was detected, although it was susceptible to several antibiotics (except methicillin, vancomycin, kanamycin, sulfamethoxazole or aztreonam), indicating that this isolate possesses the probiotic features required. Furthermore, *Pediococcus* cells show 85.71% autoaggregation, which is important to bacterial adhesion to the host intestinal epithelium. Pediococcus co-aggregation in *Salmonella typhimurium* SL 1344 (6.26%) and *E. coli* LMG 3083 (ETEC) (12.99%) is also observed, being an important characteristic in order to prevent colonization of these

Table 2
Clinical applications of *Pediococcus spp.*

Pediococcus strain	Disease	Model	Results (in comparison with control group)	Reference
P. pentosaceus LI05	Induced acute liver failure	*In vivo* Sprague-Dawley rat	↓ Alanine aminotransferase, aspartate amino- transferase, total bilirubin, histological liver abnormalities ↑ INT-γ and IL-10 level	Lv et al. (2014)
P. pentosaceus JWS939	Mice infected by *Listeria monocytogenes*	*In vitro* C57BL/6 mouse peritoneal macrophages *In vivo* BALB/c mice	*In vitro*: ↑ IL-1β *In vivo*: ↑ NO, IL-1β, and TNF-α	Choi et al. (2011)
P. pentosaceus Sn26	Induced allergic diarrhea	*In vitro* Peeyer's patch cells *In vivo* mice	*In vitro*: ↑ IL-12 and IFN-γ synthesis *In vivo*: ↓ IgE levels in serum and diarrhea symptoms	Masuda et al. (2010)
P. acidilactici M76	Induced obese mice	*In vivo* C57BL/6J mice	↑ Hepatic cholesterol and triglyceride	Moon et al. (2014)
P. pentosaceus OZF	None	*In vivo* BALB/c mice	↑ IL-6	Osmanagaoglu et al. (2013)
Synbiotic 2000[a]	Infection incidence in liver transplantation patients	*In vivo* 66 liver transplantation patients	↓ Infection incidence	Rayes et al. (2005)
Synbiotic 2000[a]	Nosocomial bacterial infections	*In vivo* patients undergoing pancreas resection	↓ Bacterial infection incidence	Rayes et al. (2007)
Synbiotic 2000[a]	Systemic inflammatory response syndrome SIRS and organ failure	*In vivo* severe acute pancreatitis patients	↓ Organ failure, septic complications and mortality	Oláh et al. (2005)
P. pentosaceus LP28	None	*In vivo*-induced obese mice	↓ Body weight, cholesterol, triglyceride	Zhao et al. (2012)
P. pentosaceus KID7	None induced hypercholesterolemia mice	*In vivo* C57BL/6J mice	↓ Serum total cholesterol, LDL cholesterol, glutamyl pyruvic transaminase	Damodharan et al. (2015)
P. pentosaceus VJ13	None	*In vitro*	↓ Cholesterol	Vidhyasagar and Jeevaratnam (2013)
P. acidilactici M76	None mice with high fat diet	*In vivo* C57BL/6J mice	↓ Hepatic cholesterol and triglyceride	Moon et al. (2014)
Synbiotic 2000[a]	Trauma patients	*In vivo* trauma patients	↓ Fewer infections, pneumonias incidence and intestinal permeability	Spindler-Vesel et al. (2007)
Synbiotic 2000[a]	Multiple trauma patients	*In vivo* multiple trauma patients	↓ Overall infection, respiratory tract infection, average days of mechanical ventilation needed, SIRS, mortality due to severe sepsis	Kotzampassi et al. (2006)

[a] Synbiotic 2000 contains 10^{10} CFU/dose of *P. pentosaceus* LMG P-20608.

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pathogenic bacteria in the epithelium. Studies in Caco-2 cells demonstrated that the adhesion factors of *P. pentosaceus* OZF cells are composed of lipid and carbohydrates compounds, lacking a proteinaceous component, which is different from other LAB. The antimicrobial effect of purified pediocin produced by *P. pentosaceus* OZF was shown in different spoilage and food-borne pathogenic bacteria (Osmanagaoglu et al., 2010).

4.2.2. Immunomodulation

Probiotic mechanisms to modulate host immune response are triggered by environmental acidification *via* organic acid and hydrogen peroxide production, inhibition of virulence factors, improvement of tight junction stability and production of antimicrobial peptides (bacteriocin) (Alexandre et al., 2014). In addition, probiotics compete for limited substrates and cellular adhesion sites with other gut bacteria including the pathogenic ones (Alexandre et al., 2014).

Immune response in BALB/c mice model was evaluated when *P. pentosaceus* OZF cells were used as a supplement (Osmanagaoglu et al., 2013). A daily *Pediococcus* dose of 10^8 CFU was administered for 30 days in BALB/c mice. *P. pentosaceus* OZF CFU recovery from mouse feces occurs up to 120 h after the first administration, displaying inhibition in *L. monocytogenes* ATCC 7644 growth. After 30 days, mice were euthanized and blood, peritoneal exudate cells (PECs) and spleen cells were collected aseptically. IL-6 synthesis in the treated group was 89.15 ± 7.89 ng/mL higher than in the control, whereas no significant difference of IL-12 levels was observed between groups. The authors also evaluated the effect of dead *Pediococcus* cells (10^7 CFU/mL) through *in vitro* experiments using PECs (200,000 cells in 200 μL) and spleen cells (4 million cells in 200 μL). No significant difference was observed regarding IL-12 and IFN-γ synthesis between the experimental and non-stimulated groups. On the other hand, only when a lower concentration of OZF cells was used (≤10^4 CFU/mL), the IL-6 production was higher in the experimental group in comparison with the control group of *Pediococcus*.

Immunomodulation is one of the probiotic properties of LAB, as antimicrobial peptides regulate gut microbiota colonization, either eliminating potential pathogenic bacteria or inducing the recruitment of specific immune cells that trigger a proper and inflammatory response (Mandal et al., 2014). In this process, anti-inflammatory cytokines (IL-10 and TGF-β) are synthesized, while pro-inflammatory cytokines (TNF e IFN-γ) are down-regulated (Mandal et al., 2014).

The effect of *P. pentosaceus* Ll05 administration in rats with acute liver failure induced by D-galactosamine (1.1 g/kg) was studied by Lv et al. (2014). Daily *Pediococcus* suspension (approximately 9.10^9 CFU) administered to rats for 8 days results in reduced levels of alanine aminotransferase, aspartate amino-transferase, and total bilirubin and decreases histological liver abnormalities in comparison to untreated rats (control). Moreover, mice supplemented with Ll05 show increased INF-γ levels, indicating an innate and adaptive immune response against infection and higher IL-10 concentration, which, in turn, is also recorded in serum analysis.

P. pentosaceus JWS939, originally isolated from duck intestine, promotes interesting immunomodulatory effects, as shown be an *in vitro* assay using C57BL/6 mouse peritoneal macrophages and *in vivo* experiments in mice infected with *L. monocytogenes* (Choi et al., 2011). In this study, all results were compared to *Lactobacillus rhamnosus* GG (LGG), a well-studied immune enhancer strain. Peritoneal macrophages (5.10^5 cells) cultivated with 100 μL of 5.10^7 CFU/mL or 1.10^7 CFU/mL of JWS939 or LGG heat-killed cells produced IL-1β in a dose-dependent manner, with higher levels recorded on *Pediococcus* treatment. Nitric oxide and tumor necrosis factor (TNF-α) concentrations are higher in macrophages incubated with JWS939 (100 μL of 5.10^7 and 1.10^7 CFU/mL, respectively) in comparison to other groups. A dose of 1.10^9 CFU of *Pediococcus* applied daily for 2 weeks in BALB/c mice results in higher levels of NO, IL-1β, and TNF-α than in mice supplemented with L. *rhamnosus* GG cells. It is relevant to note that after a lethal dose of *L. monocytogenes*, mice fed with JWS 939 survive longer than the ones fed with *Lactobacillus* cells (Choi et al., 2011).

Allergic diarrhea disease type-I is related to excessive IgE antibody production. T helper (Th) cells produce cytokines that modulate IgE synthesis: IL-4 by Th2 (induction) or IFN-γ *via* Th1 (suppression) (Masuda et al., 2010). Macrophages also play an important role, since IL-12 stimulates activation of Th1 cells. *P. pentosaceus* Sn26 cells, originally isolated from Japanese fermented vegetables, induce IL-12 and IFN-γ synthesis in Peyer's patch (PP) cells. In addition, the Sn26 strain co-cultivated with splenocytes of allergic diarrheic mice leads to strong inhibition of IgE production. Lastly, oral administration of Sn26 (1 mg) in induced allergic diarrheic mice results in reduction of both IgE serum levels and diarrhea (Masuda et al., 2010).

4.2.3. Probiotic activity in the gastrointestinal tract

Intense research focused on probiotic use has been conducted in animals and humans in order to determine alternative gastrointestinal infection treatments (Mandal et al., 2014).

Postoperative infection derived from gut bacterial translocation is commonly observed. Rayes et al. (2005) performed a clinical test in 66 liver transplantation patients. One group received Synbiotic 2000, a mix of probiotics and prebiotics including 10^{10} CFU (daily) of *P. pentosaceus* LMG P-20608, for 14 days and the other group received only fibers (prebiotics). The infection incidence was reduced for patients who received pre- and probiotics (3%), whereas infection occurred in 48% patients treated only with fiber (Rayes et al., 2005). The same approach was used by Rayes et al. (2007) in order to prevent nosocomial bacterial infections in patients undergoing pancreas resection. A group of 80 pylorus-preserving pancreato-duodenectomy patients was submitted to the same treatment as the previous study except for 8 days of pre- and probiotic administration instead of 14 days. Bacterial infection occurred in 12.5% of patients who received fiber and probiotics, while 40% of patients who consumed only prebiotics displayed nosocomial bacterial infections (Rayes et al., 2007).

Oláh et al. (2005) also verified the effect of Synbiotic 2000 in 62 severe acute pancreatitis patients. Patients who received a pre- and probiotic mix (3.0%) presented a lower rate organ failure incidence than the control group that received only fiber (17.2%).

Fermented liquid feed (FLF) in pig nutrition is composed of cereal grains (barley, wheat, oatmeal) fermented by LAB that confers several advantages over dry feed: reduction of pathogens in the gastrointestinal tract of piglets, lower incidence of clinical disease in challenge animals, and a shorter period of fecal pathogen excretion. The possibility of generating more co-products in the bioethanol industry and the political trend of antibiotic use reduction are also some benefits of FLF (Canibe and Jensen, 2012). Initially, a high ratio of enterobacteria and a low concentration of lactic acid are observed in this fermentation, and after some hours, LAB and yeasts dominate the culture, resulting in high levels of organic acids (decreased pH) and ethanol. Shlimon et al. (2006) verified that 74–88% of LAB species from barley (50%) and wheat (50%) fermented for 180 h belong to the species *P. pentosaceus*. Otherwise, *L. plantarum* species dominate FLF from cereal grain (Canibe et al., 2007). Pigs challenged with *Lawsonia intracellularis* (enteropathy) that were fed with FLF supplemented with 2.4% of LAB showed reduced pathological intestinal lesions in comparison to other groups. Moreover, fecal excretion of *Lawsonia* cells was delayed (10 days instead of 6–8 days) and displayed a shorter period (11.7 *vs.* 15.6 days) in pigs that received FLF than other diet groups (Boesen et al., 2004).

Supplementation with a mix of LAB bacteriocin producers, including *P. acidilactici* 347, in the diet of BALB/c mice decreases the presence of detrimental bacteria in the gut microbiota, such as *Staphylococcus sp.*, *Enterococcus sp.*, or *Clostridium sp.*, and increases the proportion of LAB present in the gut microbiota, not affecting its structure by the treatment (Umu et al., 2016).

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The effect of *P. acidilactici* UL5 in simulated ileal chyme was shown by Fernandez et al. (2016). *P. acidilactici* UL5 is demonstrated to be a poor competitor against gut microbiota and does not significantly decrease *L. monocytogenes* LSD 530 level. However, it is important to mention that even after three days of fermentation, this strain maintains its ability to produce pediocin PA.

4.2.4. Cholesterol level reduction

P. pentosaceus LP28, originally isolated from longan fruit, was evaluated by Zhao et al. (2012) regarding its physiological effects in high fat diet-induced mice. The test group received a high-fat diet (HFD) supplemented with *Pediococcus* cells, *L. plantarum* SN13T or any addition of LAB as a control. Mice fed for 8 weeks with HDF and daily 3.10^9 CFU LP28 cells showed reduced body weight (40%), cholesterol (70%), triglyceride (54%) and abdominal visceral fat (maintained the starting level) in comparison with the control group. Transcript analysis of lipid metabolism and biosynthesis expression genes in mice supplemented with LP28 reveals that CD36, *SCD1* (encoding stearoyl-CoA desaturase), and *PPARγ* (peroxisome proliferator-activated receptor gamma) in total mRNA liver content were lower than in the control group. CD36 is a fatty acid and oxidized low-density lipoprotein transporter that is present at low levels in normal liver tissue. Once a high-fat diet is introduced, mice have their expression of this gene enhanced, which is commonly related to hepatic fat increase and hepatic steatosis. *Pediococcus* treatment down-regulates CD36 transcription in fat diet-induced obese mice, and consequently, it could prevent hepatic steatosis.

The cholesterol-lowering activity of *P. pentosaceus* KID7 was studied by Damodharan et al. (2015) in induced hypercholesterolemia mice. Daily supplementation of KID7 (3.10^8 CFU/mL) for 32 days decreases serum total cholesterol (19.8%), LDL cholesterol (35.5%), and glutamyl pyruvic transaminase in comparison with the control group (mice without probiotic administration). Moreover, qPCR analysis revealed a relative increase of low-density lipoprotein receptor (LDLR), cholesterol-7α-hydroxylase (CYP7A1) and apolipoprotein E (APOE) mRNAs in mice treated with KID7 cells. The reduction of hepatic cholesterol might be related to CYP7A1 expression, since this enzyme catalyzes acid synthesis from cholesterol. The LDLR expression leads to higher levels of LDL absorption by hepatocytes and increases the cholesterol pool, which down-regulates LDL re-expression. Interestingly, as hepatic cholesterol is converted to bile acid by CYP7A1, the LDLR mRNA continues to be synthesized only in mice fed with *Pediococcus* cells (Damodharan et al., 2015).

In vitro studies on assimilation of soluble cholesterol (100–200 μg/mL) by five strains of *Pediococcus pentosaceus* VJ13, VJ31, VJ35, VJ49, and VJ56 cultivated in MRS broth with 0.3% bile showed 58–73% cholesterol assimilation, depending on the *Pediococcus* strain. It is worth noting that the heat-killed strain VJ13 decreases the cholesterol concentration level by 19%, as opposed to 73% in live cells (Vidhyasagar and Jeevaratnam, 2013).

Hepatic cholesterol and triglyceride level decrease in C57BL/6J mice when a high-fat diet (HFD) is administered in conjunction with *P. acidilactici* M76 cells (5.10^9 CFU/kg body weight) for 12 weeks. However, no significant differences are observed in serum lipid profile between groups. Transcript analysis of lipid metabolism-related genes reveals that acetyl-CoA carboxylase (AAC), fatty acid synthase and PPAR-γ mRNAs are lower in the HD group treated with M76 cells (HFD-PA) than in the control group (HFD), whereas carnitine palmitoyltransferase-1 (CPT-1) mRNA is higher in HFD-PA than HFD. The lipid-lowering feature of *P. acidilactici* M76 could be explained due to ACC down-regulation, as this enzyme is responsible for fatty acid synthesis, and in its absence, fatty acid oxidation is stimulated. Together, a high level of CPT-1 mRNA in the HFD-PA group is conserved and this enzyme is involved in mitochondrial fat oxidation (Moon et al., 2014).

Serum total cholesterol and LDL levels decrease in Sprague-Dawley rats treated with a mix of LAB and SAFELAC, antimicrobial peptide from *P. pentosaceus* and 5% of cellulose than in rats fed without fiber supplementation (51.7 *vs.* 73.7 mg/dL and 7.6 *vs.* 12.0 mg/dL, respectively). Fiber addition in the diet produces a 10-fold increase in the number of viable LAB in rat feces and decreases the activity of enzymes (β-glucosidase, β-glucuronidase and tryptophanase) in the intestinal microbiota that are considered harmful when they are overexpressed. The presence of 5% cellulose is more effective than 10% in all experiments, which confirms the importance of probiotic and prebiotic combination studies (Lee et al., 2011).

4.2.5. Probiotic activity in the respiratory tract

Recent studies about the beneficial effect of probiotics in gut-associated lymphatic tissue have opened a new window for respiratory infection treatment (Alexandre et al., 2014). In ventilator-acquired pneumonia (VAP), pathogenic bacteria colonize the respiratory and digestive tracts. Probiotic intake at the oropharyngeal level could replace and eliminate hazardous microorganisms or modulate the immune system's response *via* the lymphoid system of respiratory and digestive mucosa (Forsythe, 2011).

Daily use of Synbiotic 2000, a combination of pre- and probiotics (including *P. pentosaceus* LMG P-20608) previously described, in trauma patients resulted in a reduced incidence of infection and pneumonia in comparison to other treatments (glutamine; fermentable fiber; peptide diet). Furthermore, intestinal permeability decreased only in the group treated with Synbiotic 2000, from 0.148 to 0.061 lactulose-mannitol excretion ratio (Spindler-Vesel et al., 2007). Synbiotic 2000 was also the subject of a study in 65 multiple trauma patients who were randomly assigned to receive this pre-/probiotic combination or a placebo (Kotzampassi et al., 2006). Patients who received this symbiotic for 15 days exhibit a reduction, compared with the placebo-treated group, in the incidence of the following: overall infection (63% *vs.* 90%), respiratory tract infection (54% *vs.* 80%), average days of mechanical ventilation needed (15 *vs.* 26 days), systemic inflammatory response syndrome (SIRS) (49% *vs.* 77%), and mortality due to severe sepsis (14.3% *vs.* 30.0%) (Kotzampassi et al., 2006).

4.2.6. Pediococcus spp. as vaginal probiotics

LAB dominate the vaginal microbiota in healthy premenopausal women, playing a key role in combating infection by pathogens. Borges et al. (2013) isolated 35 *Pediococcus* strains that display antimicrobial activity (from 400 to 6400 AU/mL) against *L. monocytogenes*. The maintenance of vaginal acidity and the production of bacteriocin by *Pediococcus* strains prevent colonization by undesirable microorganisms. *P. pentosaceus* SB83, which lacks virulence genes, is sensitive to many antibiotics (except for tetracycline and vancomycin), possesses a weak or moderate capacity for biofilm production, and displays 6400 AU/mL antimicrobial activity against *L. monocytogenes* serotypes 1/2b and 4b and 3200 AU/mL against serotype 1/2a. It is relevant to mention that 95% of human listeriosis is caused by these three serotypes. SB83 slightly decreases in cell viability after incubation in simulated vaginal fluid at pH 4.2, but its antilisterial activity is maintained. Hence, this *P. pentosaceus* SB83 is a potential candidate to be used as a vaginal probiotic.

5. Conclusions and future trends

Highlighting the potential importance and value of bacteriocin production by *Pediococcus*, this review summarizes what is already known about *Pediococcus*-associated bacteriocins and about the use of this probiotic in food and clinical applications, thereby providing an overview of their classification, mode of action, genetic determinants, spectra of activity and factors that influence their bacteriocin production. The destiny of future *Pediococcus* application and the commercialization of these *Pediococcus*-associated bacteriocins will be dependent on whether the application will involve the use of the producing strains as probiotics or require the peptides in a (partially) purified or

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concentrated form. Currently, many *Pediococcus* strains are used as silage additives, although pediocin is still not approved as a food additive. The search for antimicrobial compounds to replace for antibiotics is one of the major challenges in the 21st century. Therefore, it is important to emphasize that it is still imperative to expand and deepen the study of *Pediococcus* as a probiotic as well as the bacteriocins produced by *Pediococcus* spp., given that they have proved to advantageously contribute to functional food properties and have presented several relevant clinical applications. The possibility of using GMO probiotics or producing recombinant bacteriocins with higher efficiency and lower cost could undoubtedly improve their chances of reaching the consumer market however regulatory issues must be addressed.

Conflicts of interest

The authors declare that they have no competing interests.

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Research review paper

Bacteriocin production by *Bifidobacterium* spp. A review

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ABSTRACT

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

Contents

1. Introduction

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

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

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

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

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

2. Antimicrobial compounds from *Bifidobacterium* spp.

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

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

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

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

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

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

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

(–): not available.

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

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

2.1. Antimicrobial compounds: production time and phases

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

2.2. Antimicrobial compounds: enzymes, pH and heat stability

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

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

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

[a] Prior to leader cleavage.

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

3. Classification of bacteriocins

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

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

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

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

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

4. Influence of culture medium and bifidogenic factors

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

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

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

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

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

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

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

5. Purification and separation

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

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

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

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

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

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

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

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

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

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

6. Conclusions and future prospects

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

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Production of bacteriocin-like inhibitory substance by *Bifidobacterium lactis* in skim milk supplemented with additives

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Bacteriocins are natural compounds used as food biopreservatives instead of chemical preservatives. *Bifidobacterium animalis* subsp. *lactis* (*Bifid. lactis*) was shown to produce a bacteriocin-like inhibitory substance (BLIS) able to inhibit the growth of *Listeria monocytogenes* selected as an indicator microorganism. To enhance this production by the strain *Bifid. lactis* BL 04, skim milk (SM) was used as a fermentation medium either in the presence or in the absence of yeast extract, Tween 80 or inulin as stimulating additives, and the results in terms of bacterial growth and BLIS production were compared with those obtained in a traditional high cost complex medium such as Man, Rogosa and Sharpe (MRS). To this purpose, all the cultivations were carried out in flasks at 200 rpm under anaerobic conditions ensured by a nitrogen flowrate of 1·0 L/min for 48 h, and BLIS production was quantified by means of a modified agar diffusion assay at low values of both temperature and concentration of *List. monocytogenes*. Although all these ingredients were shown to exert positive influence on BLIS production in both media, yeast extract and SM were by far the best ingredient and the best medium, respectively, allowing for a BLIS production at the late exponential phase of 2000 AU/ml.

Keywords: *Bifidobacterium lactis*, Bacteriocins, Skim milk, Probiotics, *Listeria monocytogenes*.

Bacteriocins are low-molecular-mass ribosomally synthetised peptides, which usually have bactericidal activity against species closely related to the producing bacteria. Bacteriocins produced by probiotics are known to inhibit food-borne pathogens such as *Listeria monocytogenes, Bacilllus cereus, Staphylococcus aureus, Clostridium perfringens*, thereby contributing to the conservation of different kinds of food products (Yildirim et al. 1999; Cheikhyoussef et al. 2009a). In recent years, the interest for probiotic bacteriocin production has constantly grown because of their use as food biopreservatives to satisfy the rising demand of consumers for natural foods without chemical preservatives.

Bifidobacteria, which proliferate in the human gastrointestinal tract, provide large health benefits to the host; therefore, they are widely used as probiotics in the industry of fermented foods. One of the main reason of their probiotic properties is the wide capacity of producing antimicrobial substances such as bifidocin B, bifidin I, bifilong and bifilact Bb-46 (Anand et al. 1984, 1985; Kang et al. 1989; Yildirim et al. 1999; Abd et al. 2004; Von Ah, 2006; Cheikhyoussef et al. 2010) and bacteriocin like inhibitory substances (BLIS) (Collado et al. 2005; Cheikhyoussef et al. 2009a). The most research has been focused on bacteriocin production by lactic acid bacteria (LAB), especially *Lactobacillus* sp. and *Pediococcus* sp. (Deegan et al. 2006), even though bacteriocins produced by *Bifidobacterium* sp. are gaining attention of the food industry because certain strains of this genus are recognised as safe (GRAS) (Picard et al. 2005), hence allowing easy application and customer acceptance.

Nonetheless, only a few studies have been done on the optimisation of bacteriocin production by *Bifidobacterium* sp., and only two review articles have been published on this issue (Cheikhyoussef et al. 2008; Martinez et al. 2013). However, these interesting reports have mainly focused on the separation and purification processes, or

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on the influence of pH and temperature, inhibitory compounds and bacteriocin structure stability rather than on the optimisation of medium composition for *Bifidobacterium* sp. cultivations (Yildirim & Johnson, 1998; Yildirim et al. 1999; Von Ah, 2006; Cheikhyoussef et al. 2009b, 2010; Lee et al. 2011). Additional significant issues that must be considered in bacteriocin production are the high cost of complex media, normally used in large scale fermentation processes such as Man, Rogosa and Sharpe (MRS) and *Bifidus* selective medium (BSM), as well as the low reproducibility and sensitivity of the agar diffusion method for the determination of bacteriocin activity (Pongtharangkul & Demirci, 2004). Thus, no previous experimental design was yet performed to optimise the fermentation process aiming at bacteriocin production by *Bifidobacterium* sp.

Based on these issues, this study was first addressed to the enhancement of bacteriocin production in flasks by *Bifidobacterium animalis* subsp. *lactis* BL 04 by exploring the separate effects on BLIS production of the addition of yeast extract, Tween 80 or inulin to skim milk (SM) selected as an alternative low-cost medium. The second aim was to select the best conditions to use in the agar diffusion bioassay for determination of BLIS production.

Materials and methods

Bacterial strains

The commercial starter freeze-dried strain *Bifidobacterium animalis* subsp. *lactis* (*Bifid. lactis*) (Danisco, Sassenage, France) was maintained in Man, Rogosa and Sharpe (MRS) medium (Sigma, St. Louis, MO, USA) and used as BLIS producer, while *Listeria monocytogenes* ATCC 13932 was maintained in brain heart infusion (BHI) medium (DIFCO, Sparks, MD, USA) containing 40% (v/v) glycerol and used as BLIS indicator microorganism. Both strains were maintained at −70 °C.

Culture media

Bifid. lactis BL 04 was grown at 37 °C either in MRS medium (DIFCO) or in skim milk (SM, DIFCO) for comparison purposes. L-cysteine 10 g/l (Sigma) and sodium thioglycolate 100 µl/l (INLAB, São Paulo, SP, Brazil) were added in both media to improve the bacteriocin production and to ensure microaerophilic conditions.

SM was prepared adding 10 g skim powder milk in 100 g distilled water and then heated at 90 °C for 10 min in water bath, model 550A (Nova Ética, Vargem Grande Paulista, SP, Brazil). Flasks were kept at 30 °C overnight to check the possible presence of contaminants.

Inoculum preparation

A single colony of *Bifid. lactis* BL 04 grown in MRS agar was inoculated into 10 ml liquid MRS medium, incubated at 37 °C for 48 h and finally used to inoculate 100 ml MRS medium contained in 250 ml Erlenmeyer flasks. After further incubation of flasks at 37 °C, cells were collected by centrifugation (4800 ***g***, 10 min, 4 °C), and pellets were washed and resuspended in sterile physiological solution (40–50 ml) until achieving an absorbance at 600 nm of about 0·9 (corresponding to about 10^8 CFU/ml).

Selection of ingredients to stimulate BLIS production

Flasks containing 50 ml SM or MRS medium were inoculated with 1000 µL of the above inoculum suspension and incubated under microaerophilic conditions in shaker at 37 °C, 50 rpm for 24 h. In order to stimulate BLIS production by *Bifid. lactis* BL 04, 1% Tween 80 (Alamar Tecno-Científica, Diadema, SP, Brazil), yeast extract (DIFCO) or inulin (Beneo-Orafti Corp., Malvern, PA, USA) were tested in triplicate for their well-known ability to improve bacteriocin expression. MRS medium and SM without additives were used as controls.

Analytical methods

Cell concentration (X) was determined by plating according to the pour-plate method and expressed as Colony Forming Units per unit volume (CFU/ml). To this purpose, serial dilutions of broth samples onto *Bifidus* selective medium (BSM, DIFCO) plus agar were made at 37 °C for 48 h in anaerobic jars.

The average specific growth rate (μ) was then calculated at the time (t) of maximum cell concentration in each run (X_m), according to the equation:

$$\mu = \frac{1}{t} \ln \frac{X_m}{X_0} \quad (1)$$

where X_0 is the starting biomass concentration, and the generation time (t_g) as:

$$t_g = \frac{\ln 2}{\mu} \quad (2)$$

The pH of the medium was measured by using means of an external pH meter (Quimis, Campanário, SP, Brazil).

Glucose and lactose concentrations were determined by high-performance liquid chromatography (HPLC). To this purpose, 10 µl aliquots of media were injected into an HPLC, model Dionex Ultimate 3000 (Thermo Fisher Scientific, Waltham, MA, USA), supplied with a sugar-separation column (Aminex-HPX-87H) as described by Mendonça et al. (2013).

For BLIS activity determination, *List. monocytogenes* was inoculated in BHI broth (DIFCO) at 37 °C and 100 rpm for 4–6 h until it reached an optical cell density of about 0·9 at 600 nm. Subsequently, 500 µl of this suspension were inoculated into 50 ml BHI agar (pre-cooled and maintained at 40 °C), aseptically laid on sterile Petri dishes and allowed to solidify. Meanwhile, fermented samples were centrifuged at 4800 ***g*** for 10 min at 4 °C, and the supernatant was filtered through filters with 0·22 µm pore

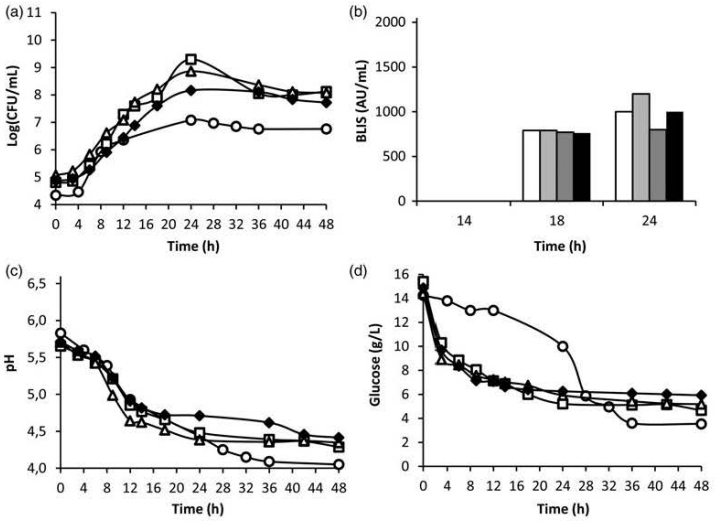

Fig. 1. Cultures of *Bifid. lactis* BL 04 carried out in shaken flasks at 37 °C and 50 rpm on MRS broth supplemented with different additives: None (○); yeast extract (□); inulin (△); Tween 80 (◆). (a) Biomass concentration (Log CFU/ml); (b) BLIS concentration (AU/ml); (c) pH; (d) glucose concentration (g/l).

diameter (Merck-Millipore, MA, USA) to avoid any possible interference of viable cells. To exclude the inhibitory effect of lactic acid, the supernatant pH was adjusted to 6·0 with 1·0 M NaOH. Finally, the cell-free culture supernatant (CFCS) was heated at 80 °C for 10 min to inactivate proteolytic enzymes. Finally, the BLIS activity was determined by the spot-on-lawn method (Pongtharangkul & Demirci, 2004), by distributing 20 µl of fermented broth, eventually diluted with phosphate buffer (10 mM, pH 6·5), onto plates containing *List. monocytogenes* ATCC 13932 as BLIS-sensitive microorganism. The BLIS activity was expressed in arbitrary units per ml (AU/ml) by the equation $AU/ml = D^n \times 1000/P$, where D is the dilution factor, n the first dilution not exhibiting any inhibition zone, and P the volume of supernatant deposited onto the agar surface.

Statistical analyses

The results were analysed by the Statistica software, version 10 (StatSoft Inc. Tulsa, OK, USA). The coefficient of determination (R^2) was used to check the effectiveness of the above model equation, and its significance tested by the Fisher's test.

Results and discussion

Different additives were tested either in SM or in MRS medium to stimulate BLIS production by *Bifid. animalis* subsp. *lactis*, namely yeast extract (YE), inulin and Tween 80.

Cultures in MRS broth

As shown in Fig. 1, the fermentation of *Bifid. lactis* in non-supplemented MRS broth gave, at the late exponential phase (24 h), a maximum biomass concentration of 7·08 Log CFU/ml, an average specific growth rate (µ) of 0·26 h^{-1} and a generation time (t_g) of 2·63 h (Table 1); however, no production of BLIS was detected as a possible result of lack of its induction.

On the other hand, in the presence of all tested additives, biomass count was always higher than 8 Log (CFU/ml), and after 24 h of fermentation reached a maximum value in MRS medium of no less than 9·30 Log (CFU/ml) when 1% of YE was added. This value is one log unit higher than that reported by Janer et al. (2004) in the same medium, whereas it agrees with those reported by Ballongue (2004) and Von Ah (2006), who detected a strong stimulating effect of yeast extract.

The addition of YE to MRS medium stimulated the growth of *Bifid. lactis* considerably, giving a 43% reduction of generation time (t_g) (1·49 h) and a 77% increase in µ (0·46 h^{-1}) compared with MRS broth without any additive. This culture, which provided the best results in terms either of growth or BLIS production, exhibited a short exponential growth phase between 4 and 24 h, after which BLIS reached its maximum level (1200 AU/ml), which appeared to be related with maximum biomass concentration. Such a BLIS activity was 78, 90 and 80% higher than those reported in the literature for Bifidocin B (Yildirim et al. 1999),

Table 1. Main parameters of *Bifid. lactis* BL 04 cultures carried out in MRS medium and skim milk (SM) either with or without different additives: Yeast extract, Tween 80, inulin

Supplement	μ (h^{-1})	t_g (h)
MRS medium		
None	$0{\cdot}26 \pm 0{\cdot}02^{a}$	$2{\cdot}63 \pm 0{\cdot}02^{h}$
Yeast extract	$0{\cdot}46 \pm 0{\cdot}01^{f}$	$1{\cdot}49 \pm 0{\cdot}01^{c}$
Inulin	$0{\cdot}40 \pm 0{\cdot}01^{d}$	$1{\cdot}73 \pm 0{\cdot}01^{e}$
Tween 80	$0{\cdot}35 \pm 0{\cdot}01^{c}$	$1{\cdot}96 \pm 0{\cdot}02^{f}$
Skim milk		
None	$0{\cdot}32 \pm 0{\cdot}01^{b}$	$2{,}16 \pm 0{\cdot}01^{g}$
Yeast extract	$0{\cdot}60 \pm 0{\cdot}02^{h}$	$1{\cdot}15 \pm 0{\cdot}02^{a}$
Inulin	$0{\cdot}49 \pm 0{\cdot}01^{g}$	$1{\cdot}41 \pm 0{\cdot}02^{b}$
Tween 80	$0{\cdot}44 \pm 0{\cdot}01^{e}$	$1{\cdot}56 \pm 0{\cdot}01^{d}$

Different letters mean statistically-significant differences according to the test of Tukey ($\alpha < 0{\cdot}05$).

Thermophicilin B67 (Von Ah, 2006) and Bifidin I (Cheikhyoussef et al. 2009a, 2010), respectively; however, none of these studies mentioned any previous screening or optimisation of the growth medium (Martinez et al. 2013). A possible explanation of the stimulating action of YE on BLIS activity is that this ingredient provides small peptides and vitamins that can be used by bifidobacteria and could be essential for antimicrobial peptides synthesis and pH regulation (Avonts et al. 2004; Jalili et al. 2010). Aasen et al. (2000) showed that an increase in YE concentration allowed remarkably enhanced bacteriocin production. Cabo et al. (2001) proposed a possible buffering power of YE, while Leroy et al. (2003) suggested that many inorganic compounds like sulphates, which are essential for the synthesis of class IIa bacteriocins and are abundant in YE, may exert positive effects over it. Even if peptides would not have benefits on the BLIS production, the vitamins present in YE could have (Cabo et al. 2001; Tomás et al. 2010).

Indeed, also inulin behaved as an ingredient able to stimulate *Bifid. lactis* BL 04 growth in MRS medium (Table 1), reaching a count of 8·86 Log (CFU/ml), $\mu = 0{\cdot}40\ h^{-1}$ and $t_g = 1{\cdot}73$ h, although its effect was weaker than that of YE. The stimulating effect of inulin on the growth of bifidobacteria was already observed by several authors (Ramirez-Farias et al. 2009; Oliveira et al. 2012). On the other hand, its stimulating effect on BLIS production at the late exponential phase (about 24 h), which could be the result of the release of fructose monomers and their subsequent assimilation through the fructose-6-phosphate shunt as an additional carbon and energy source (Van der Meulen et al. 2006; Oliveira et al. 2012), was less marked than that of YE, its activity being only 800 AU/ml. The lower biomass level and BLIS titre compared with the culture carried out in MRS supplemented with YE could be explained by the strong inhibition of growth and synthesis of bacteriocins under nitrogen starvation conditions (Leroy & De Vuyst, 2001; Tomás et al. 2010). The use of Tween 80 as a supplement led to a lower biomass concentration (8·16 Log CFU/ml) compared with YE and inulin, but BLIS activity was no less than 1000 AU/ml. Such a marked BLIS production might be related to different properties of Tween 80, among which are (a) promotion of bacteriocin agglomeration in aqueous solution by direct stimulation of protein secretion or deadsorption due to membrane alteration, (b) enforcement of bacteriocin action against the indicator microorganism by expression of binding receptors (Eijsink et al. 2002; Keren et al. 2004; Collado et al. 2005; Martinez et al. 2013), and (c) permeabilisation of the indicator microorganism membrane (Collado et al. 2005).

The progressive decrease of pH during all *Bifid. lactis* fermentations and the simultaneous decrease in glucose concentration are in agreement with the well-known heterolactic behaviour of this microorganism, while the progressive decrease of BLIS activity after 26 h of culture may be ascribed to BLIS consumption under carbon source depletion conditions.

Cultures in skim milk

In general, skim milk (SM) allowed for better growth results than MRS medium (Fig. 2a, Table 1), consistently with its content in bifidogenic factors such k-casein, casein macropeptides, N-acetylglucosamine and N-acetylneuraminic acid (Ballongue, 2004; Janer et al. 2004). Confirming the considerations already made for MRS medium, the use of YE as a supplement ensured the highest BLIS concentration (2000 AU/ml) (Fig. 2b) as well as the highest biomass production, reaching at the late exponential phase (24 h) a count of 10·9 Log (CFU/ml), $\mu = 0{\cdot}60\ h^{-1}$ and $t_g = 1{\cdot}15$ h. These kinetic growth results are in agreement with those obtained by other authors (Aasen et al. 2000; Ballongue, 2004; Janer et al. 2004; Jalili et al. 2010). Figure 3 shows, as an example, the inhibition halos produced by five aliquots of filtered fermented broth on a plate colonised by *List. monocytogtenes* selected as an indicator microorganism.

Similarly to what was observed in MRS medium, inulin exerted a weaker stimulating effect either on growth [9·9 Log (CFU/ml), $\mu = 0{\cdot}49\ h^{-1}$, $t_g = 1{\cdot}41$ h] or on BLIS activity (1600 AU/ml) than YE, while Tween 80 inhibited growth [8·8 Log (CFU/ml), $\mu = 0{\cdot}44\ h^{-1}$, $t_g = 1{\cdot}56$ h] but stimulated BLIS activity (1800 AU/ml) compared with inulin. All these BLIS activities in SM are, as an average, about twice those detected in MRS medium, which highlights the suitability of SM as an alternative medium for BLIS production.

Finally, likewise the runs in MRS, the pH progressively decreased from 6·2–6·5 to about 4·5 throughout all the runs (Fig. 2c) because of the heterolactic production of lactic and acetic acids (Gomes & Malcata, 1999; Van der Meulen et al. 2006), and BLIS activity progressively decreased after carbon source (lactose) depletion (Fig. 2d), likely due to microbial consumption of BLIS as a carbon and nitrogen source (Collado et al. 2005).

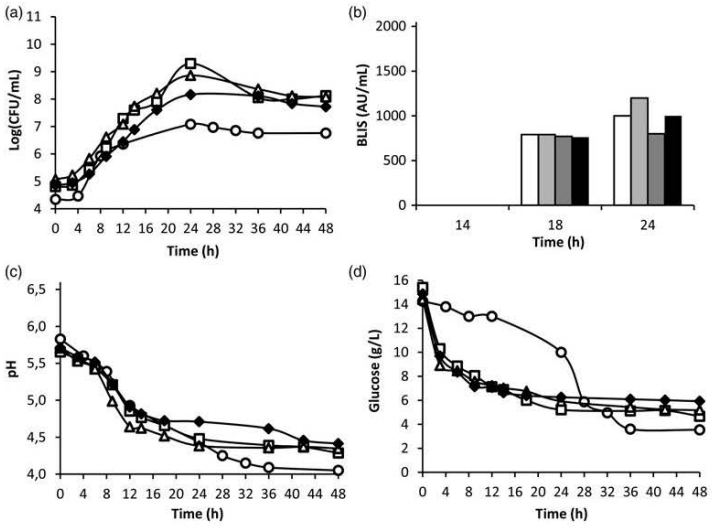

Fig. 2. Cultures of *Bifid. lactis* BL 04 carried out in shaken flasks at 37 °C and 50 rpm on skim milk supplemented with different additives: None (○); yeast extract (□); inulin (△); Tween 80 (◆). (a) Biomass concentration (Log CFU/ml); (b) BLIS concentration (AU/ml); (c) pH; (D) lactose concentration (g/l).

Fig. 3. Inhibitory activity of BLIS against *List. monocytogenes* ATCC 13932 in BHI agar.

Conclusions

This study was focused on bacteriocin production in flasks by *Bifid. animalis* subsp. *lactis* BL 04 either in MRS medium or in skim milk. The separate effects of the addition of yeast extract, Tween 80 or inulin to skim milk on BLIS production and microbial growth were investigated.

Skim milk behaved as a better medium than MRS, and yeast extract was shown to ensure the best growth and BLIS production results. For instance, in skim milk supplemented with yeast extract, cell growth [10·9 Log (CFU/ml), $\mu = 0{\cdot}60\ h^{-1}$, $t_g = 1{\cdot}15$ h] and BLIS activity (2000 AU/ml) were remarkably higher than in the presence of inulin and Tween 80. All BLIS activity values in SM were, as an average, about twice those detected in MRS medium, which highlights the suitability of SM as an alternative medium for BLIS production.

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Conflict of interest

The authors declare that have no conflict of interest.

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ORIGINAL RESEARCH

Production of bacteriocin-like inhibitory substances (BLIS) by *Bifidobacterium lactis* using whey as a substrate

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The objective of this work was to evaluate the production of bacteriocin-like inhibitory substances (BLIS) by Bifidobacterium animalis *subsp.* lactis *in whey supplemented with yeast extract, inulin, Tween-80 or L-cysteine. Cell growth, acidification, glucose and lactose consumption as well as BLIS production were measured during fermentations carried out in shake flasks. The best additive for both cell growth and BLIS production was shown to be yeast extract, which gave the highest concentrations of biomass (9.9 log cfu/mL) and BLIS (800 AU/mL). In a bench-scale fermentor,* B. lactis *growth and BLIS production were between 6% and 25% higher than in flasks depending on the conditions assayed.*

Keywords *Bifidobacterium lactis*, Whey, Bacteriocin, Anaerobic conditions.

INTRODUCTION

Whey is a by-product of high nutritional value that contains about 4.6% lactose, 0.8% protein, 0.5% fatty acids, 0.5–0.8% ash and 0.2–0.8% lactic acid (Punidadas *et al.* 1999; Antunes 2003). Given the production of cheese, 9 kg of whey is produced for each kilogram of cheese (De Wit 2001); therefore, taking into account that the cost of whey is about 10 times lower than that of traditional cultivation semisynthetic media such as de Man, Rogosa and Sharpe (MRS) medium or *Bifidus* Selective Medium (BSM), it would be of great interest to exploit this waste as a fermentation medium for the production of high value-added biomolecules such as antimicrobial compounds.

Some probiotic species of *Lactobacillus* and *Bifidobacterium* used in the manufacture of fermented dairy products can inhibit the growth of other micro-organisms, including intestinal pathogenic and spoilage bacteria, through the production of antimicrobial compounds such as bacteriocins (Cheikhyoussef *et al.* 2010; Ristagno *et al.* 2012), which offer to the producing strains an ecological advantage in the colonisation of the gastrointestinal tract (Tamime *et al.* 2005).

Bacteriocins are usually synthesised as inactive prepeptides with an N-terminal guidance sequence (Xie and van der Donk 2004), which are transported to the cell surface during the exponential growth phase and enzymatically converted into their bioactive form. The carrier contains an N-terminal peptide moiety responsible for cleavage of the guidance peptide and a C-terminal portion responsible for the hydrolysis of ATP and energy supply (Aucher *et al.* 2005).

New bacteriocins are traditionally identified by screening bacterial isolates with antimicrobial activity, followed by purification and identification of their genetic determinants. Such screening strategies are essential for the detection and identification of potent bacteriocins of various subclasses (Balciunas *et al.* 2013; Martinez *et al.* 2013).

Bifidobacteria, which are regarded as one of the predominant genera of the gastrointestinal tract (Falk *et al.* 1998), generally inhibit a wide

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range of target micro-organisms caused by the intensive release of lactic and acetic acids as products of their metabolism (Eklund 1983). Inside this genus, at least 34 species able to produce bacteriocins had been isolated from various sources, including the intestinal tract of humans (Ventura *et al.* 2007). Unlike the current situation of *Lactobacillus* spp, only a limited number of studies have been conducted on the production of bacteriocins by bifidobacteria strains.

Yildirim and Johnson (1998) isolated the first bacteriocin produced by a *Bifidobacterium* species, known as Bifidocin B, which was proven to be very effective against foodborne pathogenic micro-organisms (Yildirim *et al.* 1999). Recently, Cheikhyoussef *et al.* (2010) discovered a novel bacteriocin produced by *Bifidobacterium infantis* in synthetic (MRS) medium supplemented with L-cysteine. This bacteriocin, called Bifidin I, showed a broad spectrum of action against both Gram-negative and Gram-positive bacteria and proved to exert a strong inhibition of the pathogen *Listeria monocytogenes*.

Several compounds have been successfully employed to stimulate either bifidobacterial growth or their production of bacteriocins. Among these, inulin is a fructose-based polysaccharide whose stimulating prebiotic effect has recently been ascribed to its ability to release fructose as a carbon source (Oliveira *et al.* 2009, 2012). Tween-80 or Polysorbate 80 is a nonionic surfactant and emulsifier derived from polyethoxylated sorbitan and oleic acid, which is often used as a viscous, liquid, water-soluble yellow solution in foods and other products (O'Neil 2006). The addition of Tween-80 to culture media was shown to be very promising in bacteriocin production (Collado *et al.* 2005), being a surfactant able to increase bacteriocin concentration by making its excretion easier. In several studies on species belonging to the *Bifidobacterium* genus and even other genera performed in different culture media, yeast extract was shown to enhance cell growth. Loquasto *et al.* (2011) observed that the addition of yeast extract to whey stimulated *Bifidobacterium animalis* subsp. *lactis* (*B. lactis*) growth compared to the non-supplemented medium. Mättö *et al.* (2006) and Kiviharju *et al.* (2007) demonstrated the beneficial effect of cysteine on the growth of *B. lactis* E2010 and *Bifidobacterium longum* ATCC 15707 in MRS medium.

Based on this background, the objective of this study was to investigate the production of bacteriocin-like inhibitory substance (BLIS) by *B. lactis* HN019 using whey as an economical culture medium and evaluating inulin, Tween-80, yeast extract or L-cysteine as additives, aiming at a future large-scale use as a cheaper method to preserve foods and as a pharmaceutical alternative to nisin.

MATERIALS AND METHODS

Micro-organisms

The commercial lyophilised strain *Bifidobacterium animalis* subsp. *lactis* HN019 (*B. lactis*) (Danisco, Sassenage, France) was used in this work for bacteriocin-like inhibitory substances (BLIS) production, while *Lactobacillus sakei* ATCC 15521, *Escherichia coli* ATCC 25922 and *Listeria monocytogenes* ATCC 13932 were used as bioindicators.

Preparation of culture media

Whey powder produced from cheese whey (Cargill Agrícola SA, Campinas, SP, Brazil) was reconstituted at a concentration of 10% (w/v) and supplemented or not with inulin (Beneo-Orafti, Mannheim, Germany), Tween-80, L-cysteine (Sigma-Aldrich, St. Louis, MO, USA) and yeast extract (Synth, Diadema, SP, Brazil) at a concentration of 1% (w/v). Whey powder was reconstituted with the aid of a magnetic stirrer for 15 min and heated at 90 °C for 5 min in a thermostatically controlled bath, model 550 A (Fisatom, São Paulo, SP, Brazil). Subsequently, the suspension was immediately cooled in an ice bath and distributed into sterile Schott vials inside a laminar flow hood.

Inoculum preparation

To perform cultivations in flasks, a preculture was prepared by adding 45 mg of lyophilised stock culture of *B. lactis* into 50 mL of *Bifidus* Selective Medium (BSM) (Sigma-Aldrich) in 250-mL Schott flasks at 37 °C with magnetic agitation at 50 rpm for 24 h in anaerobic jars (BBL GasPak System; Becton Dickinson Microbiological System, Cockeysville, MD, USA).

Cultivation in shaker flasks

An aliquot of the previous culture containing approximately 10^8 cfu/mL was transferred to 250-mL Schott flasks containing 100 mL of whey, which were then incubated in a shaker at 37 °C at 50 rpm for 30 h.

Cultivation in bench-scale bioreactor

The culture for cultivations in a bench-scale bioreactor was prepared by adding 145 mg of *B. lactis* lyophilised stock culture into 150 mL of whey in 250-mL Schott flasks at 37 °C with magnetic agitation at 50 rpm for 24 h in anaerobic jars. After reaching a concentration of approximately 10^8 cfu/mL, the preinoculum was transferred to the New Brunswick Bio-Flo 115 benchtop fermentor (Eppendorf, Enfield, USA) containing 1350 mL of whey supplemented with 1% yeast extract, which was shown to be the best additive. Fermentation in the bioreactor were performed in triplicate under anaerobic conditions ensured by continuous injection of nitrogen.

Analytical techniques

Aliquots of the culture media were collected aseptically every 3 h from cultures performed either in flasks or in bioreactor, and pH, concentrations of glucose (for synthetic media), lactose (for whey), biomass, and BLIS activity were determined. Cultures were performed in triplicate, and the results were expressed as mean values.

Samples (0.1 mL) of the fermentation broth were decimally diluted six- to ninefold with sterile peptone water. Subsequently, 1.0 mL of each dilution was transferred to Petri dishes containing BSM and agar prepared in accordance with the pour plate method (Norden and Kass 1968). The plates were placed in anaerobic jars and incubated at 37 °C for 48 h. Counts were considered acceptable when the number of colonies ranged between 30 and 300 and expressed as log cfu/mL. The maximum specific growth rate (μ_{max}) was then calculated during the exponential growth phase, according to the equation $\mu_{max} = \ln(X_2/X_1)/(t_2-t_1)$, where X_2 and X_1 are the counts (cfu/mL) at time t_2 and t_1, respectively.

The pH was measured using a pH meter, model Q-400M1 (Quimis, São Paulo, SP, Brazil).

The concentrations of lactose and lactic acid were determined by high-performance liquid chromatography (HPLC) according to the method described by Donkor *et al.* (2007). Briefly, each sample was centrifuged at 15 000 *g* for 20 min using a microcentrifuge, model U-32R (Boeckel, Hamburg, Germany). The supernatant was adequately diluted, filtered through a membrane with 0.45 µm pore diameter (Millipore, Barueri, SP, Brazil), and injected into a HPLC, model Ultimate 3000 (Dionex, Sunnyvale, CA, USA), equipped with a refractive index detector, model Shodex RI-210 (Kawasaki, Kanagawa, Japan) and a HPX-87H column (Bio-Rad, Hercules, CA, USA). Runs were carried out at 35 °C, using 5.0 mM H_2SO_4 as mobile phase at a flow rate of 0.6 mL/min. High-purity lactose and lactic acid for use in HPLC (Sigma-Aldrich) were used at concentrations from 0.1 to 2.0 g/L as standard solutions to prepare the calibration curve.

Determination of BLIS activity

To detect the formation of BLIS, samples were centrifuged at 13 201 *g* at 10 °C for 10 min. The supernatant was used to quantify BLIS activity, and the pH was adjusted to 6.0 to avoid any interference in the activity tests. Samples were then heated at 90 °C for 10 min to avoid the possible influence of peroxides produced by *B. lactis* (Odamaki *et al.* 2011) and to inactivate its proteolytic enzymes. After agitation using a vortex mixer, a portion of supernatant was treated with 1 mg/mL trypsin (Sigma-Aldrich) to check the proteinaceous nature of BLIS (Todorov *et al.* 2004) and decimally diluted. Each dilution (40 µL) was placed into wells in BHI broth (Brain Heart Infusion), TSB (Trypticase Soy Broth) and MRS broth (Difco, Sparks, MD, USA) supplemented with 1% (w/w) agar, which, in turn, had been preinoculated with *L. monocytogenes* ATCC 13932, *E. coli* ATCC 25922 and *L. sakei* ATCC 15521 as indicator microorganisms, respectively. The antimicrobial activity was determined by the spot-on-lawn assay (Somkuti and Steinberg 2002; Apolônio *et al.* 2008) after incubating the plates at 35 °C. BLIS activity (AU/mL) was calculated as $2^n \times 1000$ µL/40 µL, where *n* is the final dilution showing inhibition.

Statistical analyses

All analyses were performed in triplicate, and the results were expressed as mean values. The experimental errors were expressed as standard deviation from the mean value. Values of percentage of lactose consumption, μ_{max} and lactic acid concentration were submitted to analysis of variance (ANOVA) by the Statistica 12 software (Tulsa, OK, USA).

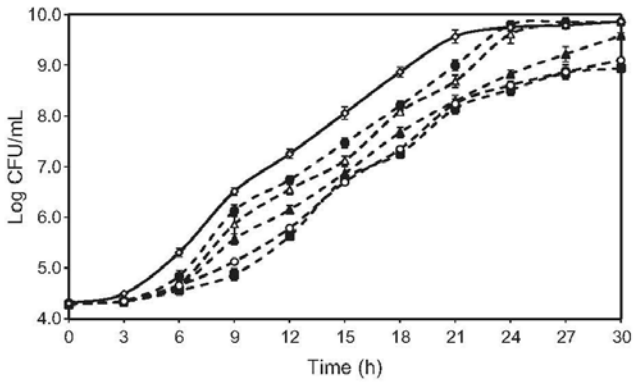

Figure 1 Biomass concentration (log cfu/mL) during *Bifidobacterium animalis* subsp. *lactis* cultivations in flasks (dashed lines). Nonsupplemented whey: (○); whey supplemented with yeast extract: (●); L-cysteine: (■); Tween-80: (▲); inulin: pH (△). Runs carried out in bench-scale fermentor (continuous lines) (◇).

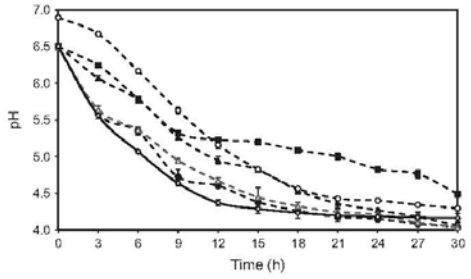

Figure 2 pH variations during *Bifidobacterium animalis* subsp. *lactis* cultivations in flasks (dashed lines). Nonsupplemented whey: (○); whey supplemented with yeast extract: (●); L-cysteine: (■); Tween-80: (▲); inulin: pH (△). Runs carried out in bench-scale fermentor (continuous lines) (◇).

RESULTS AND DISCUSSION

Cultivations in flasks

Batch cultures were performed using whey as an alternative culture medium supplemented with yeast extract (YE), L-cysteine, inulin or Tween-80, each at a concentration of 1%. These supplements were in fact shown to significantly stimulate bifidobacterial growth in previous studies (Collado *et al.* 2005; Oliveira *et al.* 2009, 2012; Loquasto *et al.* 2011). The results of these runs in terms of biomass concentration and pH along the time are illustrated in Figures 1 and 2, respectively.

At the end of the cultivation period (30 h), the pH of whey supplemented with L-cysteine was 4.5, that is 0.5 points higher than that in the presence of the other supplements. These values coincide with those reported by Loquasto *et al.* (2011) for the same micro-organism.

With respect to cell growth, the use of inulin or YE as a supplement ensured a cell concentration of *B. lactis* (approximately 9.8 log cfu/mL for both) significantly higher than those obtained with L-cysteine (8.8 log cfu/mL) or Tween-80 (9.2 log cfu/mL). Consistent with these results, when whey was supplemented with YE or inulin, the stationary phase of *B. lactis* growth started earlier (after 24 h of fermentation) compared with L-cysteine (27 h). Although whey is a natural culture medium containing nutritional substances capable of stimulating the metabolism of *B. lactis* as well as other lactic acid bacteria (Janer *et al.* 2004; Thamer and Penna 2005), it has low contents of peptides and free amino acids (Gomes *et al.* 1998) that are essential for cell growth (Antunes 2003); therefore, the strong acceleration of growth observed using YE as a supplement can possibly be attributed to its high content of these components. This stimulatory effect, already observed by Loquasto *et al.* (2011) for the same micro-organism in whey (10% v/v) supplemented with 1% YE, is consistent with the results obtained by Gomes *et al.* (1998) using hydrolysed whey supplemented with amino acids. Also, the stimulatory effect of inulin on *B. lactis* growth is in agreement with the results of a recent work, where this biopolymer was used to enhance bifidobacteria growth (Oliveira *et al.* 2009, 2012). As a result, in whey supplemented with both YE and inulin, the specific growth rate (μ_{max}) (0.60/h) was 13% higher than that obtained in nonsupplemented whey (0.53/h).

On the contrary, the addition of Tween-80 not only delayed the occurrence of the stationary phase (30 h), but also did not increase the specific growth rate of *B. lactis* when compared to whey alone.

Although *B. lactis* proved to be a bacteriocin-producing species, its BLIS activity was not very high (100 AU/mL in whey without any supplement), being *Listeria monocytogenes* the only sensitive pathogenic micro-organism among those tested. BLIS activity was 100 AU/mL with L-cysteine and Tween-80, 200 AU/mL with inulin and 400 AU/mL with yeast extract. Cheikhyoussef *et al.* (2010) and Yildirim *et al.* (1999) reported for bacteriocins produced by *Bifidobacterium infantis* BCRC 14602 and *Bifidobacterium bifidum* NCFB

Table 1 Main results of *Bifidobacterium lactis* cultivations performed in whey supplemented with different additives at a concentration of 1% (w/w). Data collected at the end of cultivations (30 h)

Supplement	*Lactose consumption (% of total)*	*Bacteriocin activity (AU/mL)**	*μ_{max} (/h)†*	*Lactic acid concentration (g/L)*
Runs in flasks				
Yeast extract	38.5 ± 0.1^{d}	400	0.60 ± 0.1^{c}	3.2 ± 0.1^{a}
L-Cysteine	36.5 ± 0.1^{ab}	100	0.55 ± 0.1^{a}	4.4 ± 0.2^{b}
Tween-80	34.8 ± 0.2^{c}	100	0.53 ± 0.1^{a}	4.6 ± 0.2^{b}
Inulin	36.2 ± 0.1^{a}	200	0.60 ± 0.2^{bc}	4.5 ± 0.1^{b}
None	36.7 ± 0.2^{b}	100	0.53 ± 0.1^{a}	4.9 ± 0.2^{bc}
Run in fermentor				
Yeast extract	40.9 ± 0.1^{f}	800	0.57 ± 0.2^{bc}	3.4 ± 0.2^{a}

*Bacteriocin activity against *Listeria monocytogenes* ATCC 13932. †Maximum specific growth rate.
Mean values (n = 3) ± standard deviations with different letters in the same column mean that they significantly differ among values of the same parameter ($P < 0.05$).

1454, both in cell-free supernatants, antimicrobial activities of 1600 and 3200 AU/mL, respectively. It is noteworthy that these researchers used MRS broth supplemented with 0.05% (w/v) L-cysteine hydrochloride, that is a medium much more expensive than whey used in this study. On the other hand, Bendali *et al.* (2008) and Bizani *et al.* (2005) reported values of antimicrobial activity similar to those obtained in this work, but the micro-organisms employed were *Lactobacillus paracasei* subsp. *paracasei* and *Bacillus cereus* 8A, both cultured in BHI medium. In all these previous studies, and in this current work, the sensitive indicator micro-organism was always *L. monocytogenes*. Regarding the heat stability, BLIS was shown to be stable in temperatures ranging from 80 to 100 °C (results not shown), confirming the findings of previous studies (Cheikhyoussef *et al.* 2009; Yildirim *et al.* 1999).

The influence of the above additives on the production of lactic acid was contrary to that on BLIS activity, in that its highest concentration at the end of cultivations was observed in nonsupplemented whey (4.90 ± 0.16 g/L) and the lowest one in whey supplemented with YE (3.23 ± 0.12 g/L), conditions under which *B. lactis* showed the minimum (100 AU/mL) and maximum (400 AU/mL) antimicrobial activities, respectively (Table 1).

Even though the percentage of lactose consumption varied slightly (from 34.8 to 38.5%) from one culture to another carried out at the same initial concentration (53.0 g/L), these results as a whole suggest that BLIS production is linked to the consumption of the carbon source. According to Gui and Li (2013), lactose consumption occurs when bacteriocin and lactic acid accumulate in the culture medium, which indicates that this sugar can be used to support the production of both. One can therefore infer that BLIS produced by *B. lactis* is a primary metabolite (Hugas *et al.* 2002) resulting from a type II fermentation according to the classification of Gaden (1959).

Cultivation in bioreactor

Fermentation was also carried out in a bench-scale fermentor using whey containing 10% (w/v) total solids supplemented with 1% (w/v) YE, because in flasks these were the conditions showing the highest biomass concentration at the end of the exponential growth phase and, at the same time, the highest BLIS activity. This experiment was performed in triplicate under conditions of temperature (37 °C) and agitation (50 rpm) that simulated at the best of those in flasks.

As can be seen in Figure 1, under these conditions, *B. lactis* showed higher and accelerated growth compared to cultivations in flasks. When grown in fermentor, the micro-organism did in fact reach the exponential growth phase, on average, 3 h faster, and the cell concentration (9.57 log cfu/mL) was significantly higher than that obtained in flasks (8.96 log cfu/mL). This result may be related to the fact that the conditions occurring in flasks are microaerophilic, while *B. lactis* is notoriously a strictly anaerobic micro-organism. Therefore, low agitation and injection of nitrogen were sufficient to homogenise the culture medium and promote an anaerobic fermentation, respectively. The percentage of lactose consumption was consequently higher in fermentor (40.9%) compared to flasks (38.5%).

As far as the acidifying capacity is concerned, it can be observed in the same figure that the pH behaviour was similar in flasks and bench-scale fermentor.

B. lactis growth in whey supplemented with YE was higher than that found after 24 h of culture (7.16 log cfu/mL) by Jalili *et al.* (2010), who cultivated the wild-type strain of the same micro-organism in the same culture medium, but with a slightly more intense agitation rate (60 rpm).

BLIS activity obtained in bench-scale fermentor using whey supplemented with YE was 100% higher (800 AU/mL) than that in flasks (Table 1).

CONCLUSIONS

Whey supplemented with yeast extract or inulin ensured the highest growth of *B. lactis* in shake flasks compared either with whey supplemented with L-cysteine or without any supplement. The addition of yeast extract led to a significant increase in the production of bacteriocin-like inhibitory substance (BLIS), thus proving to be an essential medium ingredient for cultures. In a bench-scale fermentor, *B. lactis* proved to be a promising bacteriocin producer, exhibiting a BLIS activity (800 AU/mL) under anaerobic conditions that was 100% higher than in flasks. As whey is much cheaper than traditional semisynthetic media, it may reduce the cost of BLIS production, making its use more competitive. In addition, the strain of *B. lactis* employed in this work showed antimicrobial activity against the pathogenic species *Listeria monocytogenes*, and the BLIS produced appeared to have similarity with Bifidin, even though more studies are needed to confirm this conclusion. It is however necessary to optimise BLIS production by bifidobacteria, as in the case of Bifidin I, which has a broad spectrum of action against both Gram-positive and Gram-negative bacteria.

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Review

Overview of *Lactobacillus plantarum* as a promising bacteriocin producer among lactic acid bacteria

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ABSTRACT

Chemical preservatives have been traditionally used during the manufacturing of processed products. However, the continuous growing interest of consumers for fresh and natural products makes it necessary to search for alternative compounds. In this context, food industries have been widely using lactic acid bacteria (LAB) as natural preservatives, due to their ability to produce antibacterial compounds such as bacteriocins. Similarly, pharmaceutical industries have improved the use of these bacterial peptides, with antibacterial activity, trying to reduce the indiscriminate use of antibiotics in food products for human and animal consumption. Among LAB, *Lactobacillus plantarum* can be adapted to various niches thanks to its ability to ferment a wide range of carbohydrates. Additionally, it can be used as starter culture in food fermentations and as an ingredient for probiotic foods, contributing to the organoleptic characteristics of foods at the same time prolonging the shelf-life and safety of these products. The amount of valuable substances obtained from *L. plantarum* species isolated from different ecological niches is also worth noting, thus proving it to be one of the most important and versatile species among LAB.

Contents

1. Introduction

Industrialized food has experienced in recent years an increase in the use of chemical additives in their formulations; however, consumers are becoming more and more worried about using these chemical

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additives in their diet. For this reason, there is a strong trend for seeking natural and fresh foods, free of chemical preservatives. This fact, associated with the growing demand for minimally processed foods, has encouraged the search for effective natural preservatives, among which, antibacterial compounds such as bacteriocins, fulfill these requirements (Castro, Palavecino, Herman, Garro, & Campo, 2011).

In order to control these facts, an alternative would be to use bacterial peptides with antibacterial activity, such as bacteriocins (Parada, Caron, Medeiros, & Soccol, 2007).

Bacteriocins are peptides or proteins synthesized within ribosomes and released into the extracellular medium by Gram-positive and Gram-negative microorganisms, although, those produced by lactic acid bacteria (LAB) have received greater attention, in recent years, due to their high potential for application in the food industry as natural conserving agents (Leroy & De Vuyst, 2004). Among LAB, the largest group is the genus *Lactobacillus*, which comprises more than 150 different species (Siezen, Tzeneva, Castioni, Wels, Phan, Rademaker, et al., 2010). Some of those are associated with the promotion of health benefits to the host (Bosch et al., 2011). Among them, *L. plantarum* can be pointed out as an industrially important microorganism that can be found and isolated from dairy products and fermented foods such as sauerkraut, sourdough, sausages, cheeses, wines, olives and pickled vegetables from environments such as cow-dung, silage and from sewage; as well as from the human mouth, intestinal tract and stools (Hammes & Vogel, 1995; Parente, Ciocia, Ricciardi, Zotta, Felis & Torriani, 2010).

In addition, certain *L. plantarum* strains have probiotic properties, which have been used for the development of functional foods and potential oral vaccines (Parente et al., 2010). To carry out such probiotic activities, the strains should have the ability to produce substances such as bacteriocins, which offer advantages in colonization and competition in the gastrointestinal tract (Castro et al., 2011). Additionally, these compounds have bactericidal or bacteriostatic action on pathogenic bacteria, which includes important pathogens such as *Listeria monocytogenes*, *Clostridium botulinum* and *Staphylococcus aureus* (De Vuyst & Leroy, 2007). Recently, the food industry has shown an increasing interest in the use of bacteriocins as a replacement for chemical preservatives, as they are effective at low concentrations and, when added to food, do not alter its sensory quality (Zacharof, Coss, Mandale, & Lovitt, 2013).

In this context, the purpose of this work is to provide, as a first step, a general overview of LAB along with the bacteriocins produced by these strains, before addressing a detailed study of the *L. plantarum* species, itemizing the bacteriocins produced up to now, with the aspect concerning their biosynthesis, their main applications, mainly in the food and pharmaceutical industries, and the purification considered in order to obtain a deeper knowledge of these peptide bacteriocins.

2. General characteristics of LAB

The group of LAB associated with food includes 11 genera: *Carnobacterium*, *Enterococcus*, *Lactococcus*, *Lactobacillus* (by far the most investigate genus), *Lactosphaera*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Vagococcus*, and *Weissella* (Mogensen, Salminen, & O'Brien, 2003; Vries, Vaughan, Kleerebezem, & Vos, 2006).

These microorganisms are Gram (+), non-sporulating, catalase-negative, acid-resistant, $pH_{optimum}$ for growing between 4.0 and 4.5, anaerobic aerotolerant, $T_{optimum}$ for growing is 30 °C (mesophilic) or 42 °C (thermophilic) and can have different shapes, like rods (bacilli) and a sphere (coccus) (Todorov & Franco, 2010).

Lactobacillus plantarum is a facultative heterofermentative lactic acid bacterium, which ferments carbohydrates generally by the phosphoketolase pathway (PKP). The fermentation of pentoses (xylose, ribose) leads to the formation of pyruvate and acetyl-P and their subsequent conversion to lactate and acetate, respectively. Hexoses (glucose, fructose, mannose) in these bacteria can be converted to lactate, CO_2, and ethanol (Hammes & Vogel, 1995; Mayo et al., 2010; Todorov & Franco, 2010). In addition, its genome encodes all enzymes required for the glycolysis and phosphoketolase pathways (Kleerebezem, Boekhorst, Kranenburg, Van Molenaar, Kiupers, Leer, et al., 2003). *L. plantarum* has broad versatility, insofar as it can be found in many ecological niches, as well as in human and animal gastrointestinal tracts (Siezen et al., 2010). The ability to inhabit different niches is associated with its ability to ferment a variety of sugars (Prins, Botha, Botes, Kwaadsteniet, Endo & Dicks, 2010). They have developed very efficient transport systems, which enable them to quickly take up the necessary solutes (Salminen & Von Wright, 1993). Particularly, this flexible and adaptive behavior of *L. plantarum* is reflected by the relatively large number of regulatory and transport functions, including 25 complete sugar phosphotransferase systems (PTSs) (Kleerebezem et al., 2003).

LAB are used in the food industry due to their ability to inhibit or reduce contamination by spoilage and/or pathogen microorganisms through the production of various antimicrobial compounds (Martinez, Balciunas, Salgado, et al., 2013). The acidification of food – mainly by lactic acid formation – is probably the primary factor in the inhibition of undesirable microorganisms. LAB reduces the pH to values close to 4 in these foods, which hinders the survival of microorganisms sensitive to acid medium. Such condition leads to the increase of the fermented product shelf life, when compared to the non-fermented one (Leroy & De Vuyst, 2004).

These bacteria are still used in the food industry for the development of organoleptic properties of fermented foods (Carminati, Giraffa, Quiberoni, Binetti, Suárez & Reinheimer, 2010; Todorov, LeBlanc, & Franco, 2012). This occurs due to the large number of glycolytic, lipolytic and proteolytic enzymes that transform some medium nutrients into compounds with sensory properties, which, in the end, gradually modify the structure and aroma of the fermented food (Todorov et al., 2012). In addition, LAB are also used as probiotic starter cultures (Carminati et al., 2010).

3. Bacteriocins from LAB

LAB-producing bacteriocins (antimicrobial peptides synthesized in ribosomes) kill bacteria at much lower concentrations than eukaryotic antimicrobial peptides, probably because they interact with a specific receptor present on target cells (Cotter, Hill, & Ross, 2005; Drider, Fimland, Héchard, McMullen, & Prévost, 2006). Some studies indicated that the 35d-plantaricin bacteriocin produced by *L. plantarum* 35d showed to be active against *Aeromonas hydrophila*. Meanwhile bacteriocins ST28MS and ST26MS, produced by *L. plantarum* isolated from syrup inhibited the growth of *Escherichia coli*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* and other Gram-positive microorganisms (Messi, Bondi, Sabia, Battini, & Manicardi, 2001; Todorov & Dicks, 2004).

The bacteriocin production often occurs during late log phase or early stationary phase, and is generally influenced by a quorum sensing mechanism or by any sign of stress (Martinez, Balciunas, Converti, Cotter, & Oliveira, 2013). They differ from the majority of antibiotics due to their molecular proteinaceous constitution, being rapidly degraded by proteases in the human digestive tract (Parada et al., 2007).

3.1. History of bacteriocins

The first report of an antibacterial substance was conducted in 1925, when André Gratia published an article regarding the inhibitory capacity of *E. coli* on other strains of the same species. The produced compound, considered as responsible for the inhibitory effect, was called "colicin", in reference to the producer microorganism (Collins, Cotter, Hill, & Ross, 2010). In 1928, the ability of certain *Lactococci* strains to exert inhibition on other LAB strains was reported, and later, in 1947, Mattick and Hirsh concentrated an inhibitory substance isolated from a strain of *Lactococcus lactis* subsp. *lactis*, termed nisin (Cotter et al., 2005). This bacteriocin was initially purified and marketed in

1953 in England and then, in 1969, was considered to be safe for use in food products by the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives. It was also in 1953 that the term "bacteriocin" was proposed for antimicrobial peptides produced by microorganisms (Reeves, 1965). In 1983, in Europe, nisin was added to the list of food additives and, in 1988, the American Food and Drug Administration (FDA) authorized its use in processed cheeses (Collins et al., 2010). Although nisin is the only bacteriocin approved by the FDA for use in foods, pediocin, attained from strains of *Pediococcus acidilactici*, *Pediococcus parvulus* and *L. plantarum* WHE92, has also been employed as a preservative in industrialized foods (Enan, Essawy, Uyttendaele, & Debevere, 1996; Wang & Wang, 2014). Indeed, there is a commercial pediocin – ALTA 2341®, produced by Quest International, Sarasota, Florida from *P. acidilactici* – that is used as a medium component for fermentation processes (Papagianni & Anastasiadou, 2009). As ALTA 2341® presented high inhibitory action on *L. monocytogenes*, the producer applied for its approval by FDA (Chen, Sebranek, Dickson, & Mendonca, 2004).

A remarkable achievement on bacteriocins was attained in 1994, when a strain of lantibiotic called plantaricin C from *L. plantarum* LL441, was isolated from ripening cheese (González, Arca, Mayo, & Suárez, 1994).

3.2. Classification of bacteriocins

In spite of bacteriocins from lactic acid bacteria (LAB) differing in their spectrum of activity and in biochemical and genetic determinants, some common characteristics allow them to be divided into four classes, based on primary structure, molecular weight, heat stability and molecular organization (Cotter et al., 2005; Heng, Wescombe, Burton, Jack, & Tagg, 2007):

- Class I (lantibiotics): consists of linear (type A) and globular (type B) peptides, with low molecular weight (<5 kDa, with approximately 19 to 38 amino acids). They are post-translational modified peptides that contain unusual amino acids such as lanthionine and derivatives (Cintas, Casaus, Herranz, Nes, & Hernández, 2001; Drider et al., 2006; Todorov, 2009). Nisin is the first and the most well-known lantibiotic (Ghrairi, Chaftar, & Hani, 2012);
- Class II (non-lantibiotics): composed of thermostable peptides (<10 kDa, with approximately 37 to 48 amino acids), which, according to Drider et al. (2006) are divided into three subclasses known as Class IIa (group of pediocin-like bacteriocins active against *Listeria* spp.), Class IIb (bacteriocins requiring the union of two peptides to completely exert antibacterial effect, e.g., lactocin G) and Class IIc (bacteriocins which have a covalent bond between the C and N terminal, resulting in a cyclic structure) (Balciunas et al., 2013);
- Class III: represented by thermolabile peptides of high molecular weight (>30 kDa), such as helveticin J, acidophilucin A, and lactacins A and B (Heng et al., 2007);
- Class IV: composed of complex bacteriocins that contain carbohydrate or lipid moieties, in addition to the protein portion (Heng et al., 2007). However, Cleveland, Montville, Nes, & Chikindas (2001) proposed that these complexes are artifacts of partial purification and not a new class of bacteriocins.

Among all of these peptide bacteriocins produced by LAB, subclass IIa has arisen as one of the most interesting groups for use in food preservation (inhibiting the growth of gram-positive food spoilage and pathogenic bacteria such as *Bacillus cereus*, *Clostridium perfringens*, *S. aureus*, and *L. monocytogenes*) as well as in medicine (as antibiotic complements in treating infectious diseases or as antiviral agents) (Drider et al., 2006). Some bacteriocins produced by *L. plantarum* have been ascribed to this group, including plantaricin 423 produced by *L. plantarum* strain 423. This bacteriocin was able to inhibit the growth of *L. monocytogenes* in ostrich salami meat (Dicks, Mellett, & Hoffman, 2004).

Other plantaricins, such as EF, JK, NC8 and J51, were cataloged into the two-peptide bacteriocins (subclass IIb) (Anderssen, Diep, Nes, Eijsink, & Meyer, 1998; Diep, Havarstein, & Nes, 1996; Diep, Straume, Kjos, Torres, & Nes, 2009). Their activity depends, by definition, on the complementary action of two different peptides, where their cationic nature is essential, facilitating the initial contact between bacteriocins and the negatively charged membranes via electrostatic interactions (Diep et al., 2009).

Finally, plantaricin A, a one-peptide bacteriocin without post-translational modifications, is included in subclass IIc (Diep et al., 2009). The antimicrobial spectrum of plantaricin A is relatively narrow, comprising mainly different *Lactobacillus* species (*Lactobacillus casei*, *Lactobacillus sakei*, *Lactobacillus viridescens* and *L. plantarum*). The antimicrobial activity of plantiricin A was 10–100 fold lower than plantiricins EF and JK (Anderssen et al., 1998).

3.3. Biosynthesis and mode of action of bacteriocins

According to Drider et al. (2006), at least four genes are needed for the production and secretion of bacteriocins. In particular, they are (i) the structural bacteriocin gene, encoding a prebacteriocin; (ii) the immunity gene, encoding an immunity protein that protects the bacteriocin producer from its own bacteriocin; (iii) a gene encoding an ABC (ATP-binding cassette) transporter necessary for secretion; and (iv) a gene encoding an accessory protein of unknown function. Bacteriocins can result from the expression of a gene located at the chromosome (plantaricin ST31 for example) or at a plasmid (plantaricin 423 for example) (Reenen, van Dicks, & Chikindas, 1998; Todorov, 2009; Todorov et al., 1999). However, when two bacteriocins are produced by a strain one can be chromosomal, such as carnobacteriocin BM1, and the other from the plasmid, such as carnobacteriocin B2 (Todorov, 2009). Of course, there are situations in which two or more bacteriocins can only originate either from the chromosome or plasmid (Todorov, 2009).

Most class II bacteriocins are synthesized primarily in the form of a pre-peptide or a biologically inactive pre-bacteriocin. This compound contains a sequence from 18 to 27 amino acids presenting two glycines at the N-terminus. This sequence has the function of preventing the bacteriocin to be activated inside the producer cell and serves as recognition signal for the transport system involving the ABC transporter proteins and accessory protein (Nes, Diep, Havarstein, & Brurberg, 1996; Savadogo, Ouattara, Bassole, & Traores, 2006). The two glycines present in the sequence are responsible for recognition by the pre-bacteriocin transport system (Moll, Konings, & Driessen, 1999). After recognizing the pre-peptide, the leader amino acid sequence of bacteriocin is removed and then the active peptide/bacteriocin is secreted into the extracellular medium (Ehrmann, Remiger, Eijsink, & Vogel, 2000).

Regarding the mode of action, different mechanisms have been proposed for bacteriocins. Such mechanisms directly depend on factors related to bacterial species and their growth conditions, bacteriocin dose employed and purification degree (Parada et al., 2007). In particular, these mechanisms can promote a bactericidal effect, with or without cell lysis, or bacteriostatic, inhibiting cell growth (Cintas et al., 2001). Usually, pore formation – which results in the variation of the cytoplasm membrane potential due to the hydronium ion exchanging between the inner and outer membrane surfaces – is the main mechanism by which most of the bacteriocins from LAB exert their antibacterial effect (Ghrairi et al., 2012). In class II, this mechanism is triggered when bacteriocin binds to a protein-receptor on the cell membrane of the target bacteria, although some authors believe that such protein-receptor does not appear to be essential for binding (Chen, Shapira, Eisenstein, & Montville, 1997; Jack, Tagg, & Ray, 1995).

Although it has been reported that bacteriocins show a bactericidal mode of action centered against homologous species, some of them

have similar activity against food-borne pathogens; including the bacteriocin produced by *P. acidilactici* M that inhibits a large number of bacteria, such as *S. aureus*, *L. monocytogenes*, *C. perfringens*, *Bacillus coagulans*, *B. cereus*, and *A. hydrophila*; or the bacteriocin sakacin C2 produced by *L. sakei* C2a that inhibits many Gram-positive and Gram-negative bacteria (Hu, Zhao, Zhang, Yu, & Lu, 2013).

Nevertheless, the antimicrobial activity of bacteriocins is unstable and inconsistent, as it depends on the chemical and physical conditions of foods. There are factors that can interfere on the bacteriocin production by LAB such as unsuitable process conditions (pH, temperature, nutrients, among others), spontaneous cell loss on producing bacteriocin, infection of the cell by phage and the presence of competitive microorganisms in the medium (Schillinger, Geisen, & Holzapfel, 1996). Besides, the bacteriocin effectiveness would also be affected by the presence of bacteriocin-resistant microorganisms, enzymes (like proteases), occurrence of oxidation-reduction reactions, interaction with components of the food formula (fats, proteins, preservatives, pH, for instance) and diffusion restraints due to high salt concentration. Additionally, it can be influenced by the presence of nitrate and nitrite and low water activity, which can lead to inadequate distribution of the bacteriocin throughout the food product (Alves, Martinez, Lavrador, & De Martinis, 2006; Schillinger et al., 1996).

According to Kristo, Koutsoumanis, & Biliaderis (2008), bacteriocins can present a higher effectiveness when added into films and not directly incorporated to the product. In fact, the production of bacteriocin by *L. plantarum* was higher in cellulose derivative films when compared with protein films (Sánchez-González, Saavedra, & Chiralt, 2013).

The mode of action of plantaricin C, a bacteriocin produced by strains of *L. plantarum*, depends on the target microorganism, showing a different behavior against Gram-positive bacteria (González, Arca, Mayo, & Suárez, 1994). Thus, this bacteriocin showed a bactericidal mode of action, with the absence of concomitant or subsequent cell lysis, after being added to *L. sakei* CECT 906, reducing 50% the viability of exponentially growing cultures after 1 h without absorbance reduction. Meanwhile, it was observed that a bacteriolytic mode of action against *Lactobacillus fermentum* LMB 13554 decreases the viability of culture to 0.6% in only 5 min, accompanied by a drastic lowering in the optical density. Additionally, it showed complete lysis against *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG 13551 and almost 100% viability reduction without an apparent decrease in optical density using *Lactobacillus helveticus* LMG 13555 and *Leuconostoc mesenteroides* subsp. *cremoris* NCDO 543. According to these authors, the lytic effect of plantaricin C was not observed with most LAB bacteriocins being potentially useful in accelerated food processing, releasing the enzymes contained in the starters into their substrates.

Other plantaricins show a broader antimicrobial spectrum inhibiting food-borne pathogens in addition to the closely related *Lactobacilli* species. For example, partially purified plantaricin 163, produced by *L. plantarum* 163 isolated from traditional Chinese fermented vegetables, inhibits Gram-positive bacteria (*S. aureus*, *L. monocytogenes*, *Bacillus pumilus*, *B. cereus*, *Micrococcus luteus*, *Lactobacillus thermophilus*, and *Lactobacillus rhamnosus*) and Gram-negative bacteria (*E. coli*, *P. aeruginosa*, and *Pseudomonas fluorescens*). However, it is unable to show antimicrobial activity against fungi such as *Penicillium notatum*, *Aspergillus niger*, and *Rhisopus nigricans* (Hu et al., 2013). Plantaricin LP84 showed activity against Gram-positive, Gram-negative, food-borne pathogenic, and spoilage bacteria (Suma, Misra, & Varadaraj, 1998). Plantaricin UG1 inhibits strains of the genera *Lactobacillus* and *Lactococcus*, in addition to food-borne pathogens such as *L. monocytogenes*, *B. cereus*, *C. perfringens*, and *Clostridium sporogenes* (Enan et al., 1996). Meanwhile, plantaricin MG presented inhibitory activity against Gram-positive and Gram-negative bacteria including *L. monocytogenes*, *S. aureus*, *Salmonella typhimurium*, and *E. coli* (Gong, Meng, & Wang, 2010).

4. *L. plantarum*

L. plantarum is one of the most widespread species of the genus *Lactobacillus* and is being widely used in food-related technologies (Brinques, Peralba, & Ayub, 2010; Sauvageau et al., 2012). This microorganism is a facultative heterofermentative LAB (Group II) (Bove et al., 2012; Siezen & van Hylckama Vlieg, 2011). It is acid tolerant and considered as a safe microorganism (*Generally Regard as Safe* – GRAS) (Brinques et al., 2010). As it is a heterogeneous species, it is closely associated with the species *Lactobacillus pentosus*, *Lactobacillus paraplantarum* and, more recently, *Lactobacillus fabifermentans* (Parente et al., 2010; Siezen & van Hylckama Vlieg, 2011). This relationship was identified when more than 99% of their rRNA presented identical sequences, suggesting high phenotypic and genotypic similarity between species (Parente et al., 2010).

L plantarum is the most important and versatile species of the group, and can be found as part of the microbiota of starchy foods and cereals, meats, dairy products, vegetables, fruits and drinks (Ricciardi et al., 2012). According to Todorov et al. (2011), different strains have been isolated from various niches, such as fermented milk, cheese, fermented cucumber, fermented olives, pasta, pineapple, grapefruit juice, sorghum beer and barley, molasses, boza, kefir and amasi. These strains have proven to be able to survive gastric transit and colonize the intestinal tract of humans and other mammals, and are considered as a member of the natural microbiota of these niches (Kleerebezem et al., 2003; Mathara et al., 2008). Some authors report that *L. plantarum* can adapt to various niches due to its ability to ferment a wide range of carbohydrates (Brinques et al., 2010; Prins et al., 2010; Todorov, 2008).

Furthermore, *L. plantarum* has traditionally been used in starter cultures in food fermentations (Bove et al., 2012) and also as an ingredient for probiotic foods, such as the *L. plantarum* 299v strain, which is widely marketed (Siezen & van Hylckama Vlieg, 2011).

4.1. Bioproduction of bacteriocins from L. plantarum

Several studies have been focused on the optimization of culture medium and growth conditions in order to increase the production of bacteriocins by *L. plantarum*. There are many works describing the production of bacteriocins by strains of *L. plantarum*, namely: *L. plantarum* ST194BZ (Todorov & Dicks, 2005), *L. plantarum* ST13BR (Todorov & Dicks, 2004), *L. plantarum* ST414BZ (Todorov & Dicks, 2006b), *L. plantarum* ST664BZ (Todorov & Dicks, 2006b), *L. plantarum* ST23LD (Todorov & Dicks, 2006a), *L. plantarum* ST341LD (Todorov & Dicks, 2006a), *L. plantarum* AMA-K (Todorov, Nyati, Meincken, & Dicks, 2007), *L. plantarum* ST26MS, *L. plantarum* ST28MS and *L. plantarum* ST32 (Todorov, 2008; Todorov, Gotcheva, Dousset, Onno, & Ivanova, 2000; Todorov, Powell, Meincken, Witthuhn, & Dicks, 2007; Todorov, Van Reenen, & Dicks, 2007).

Despite the great number of studies carried out until this moment, there is little knowledge about the growth conditions required for optimal production of bacteriocins by *L. plantarum*, and an ideal fermentation process has not been established (Todorov, Van Reenen, & Dicks, 2004). It is known that cell growth of *Lactobacillus* is directly influenced by the conditions of pH, temperature, medium composition, and aeration rate, among other factors. Since LAB are quite demanding on nutritional requirements, a rich medium is extremely necessary for good growth (Brinques et al., 2010).

4.2. Bacteriocins produced by L. plantarum

A wide variety of bacteriocins produced by different *L. plantarum* strains have been isolated and described. Table 1 shows some examples of bacteriocins produced by *L. plantarum* isolated from various fermented products, their respective biochemical features and some available genetic information. The examples are detailed as follows:

APÊNDICE 7. Overview of Lactobacillus plantarum as a promising bacteriocin producer among lactic acid bacteria

Table 1
Some examples of bacteriocins produced by *Lactobacillus plantarum* isolated from various ecological niches.

Isolation niche	Strain name	Bacteriocin produced	Biochemical features	Genetic information	Some pathogens inhibited	Reference
Meat	*L. plantarum* UG1	Plantaricin UG1	Single-peptide with molecular mass between 3.0 and 10.0 kDa	Chromosomally encoded	*L. monocytogenes*, *B. cereus*, *C. perfringens* and *C. sporogenes*	Enan et al. (1996)
	L. plantarum 35d	Plantaricin 35d	Single-peptide with molecular mass estimated to be 4.5 kDa	Information about genetic determinates not shown	*S. aureus*, *L. monocytogenes* and *A. hydrophila*	Messi et al. (2001)
	L. plantarum LT154	Plantacin 154	Single-peptide peptide with molecular mass estimated to be 3.0 kDa or less	Plasmid-encoded	*Enterococcus faecalis*, *Bacillus* spp., *Staphylococcus* spp. and *S. typhimurium*	Kanatani and Oshimura (1994)
	L. plantarum SA6	Plantaricin SA6	Single-peptide peptide with molecular mass estimated to be 3.4 kDa	Information about genetic determinates not shown	*L. plantarum*, *Lactobacillus brevis*, *Leuconostoc* spp. and *Listeria grayi*	Rekhif et al. (1995)
	L. plantarum ST202Ch ST216Ch	BacSt202Ch and bacST216	Single-peptide with molecular mass estimated to be 3.5 and 10 kDa, respectively	Chromosomally encoded	*Enterococcus faecium*, *E. coli*, *L. monocytogenes*, *Pseudomonas* spp. and *S. aureus*.	Todorov et al. (2010)
Fish	*L. plantarum* PMU 33	Plantaricin W	Two-peptide (α and β-peptide) with molecular masses estimated to be 3.2 and 3.0 kDa, respectively	Chromosomally encoded	*L. monocytogenes*, *B. cereus*, *S. aureus*, *E. faecium* and *E. faecalis*.	Noonpakdee et al. (2009)
	L. plantarum BF001	Plantaricin F	Single-peptide with molecular mass between 0.4 and 6.7 kDa	Information about genetic determinates not shown	*S. aureus*, *S. typhimurium*, *L. monocytogenes* and *P. aeruginosa*.	Fricourt et al. (1994)
Fruits and vegetables	*L. plantarum* ST28MS and ST16MS	ST28MS and ST16MS	Single-peptide with molecular masses estimated to be 5.5 and 2.8 kDa, respectively	Chromosomally encoded	*L. sakei*, *S. aureus*, *E. faecalis*, *P. aeruginosa*, *E. coli* and *A. baumanii*	Todorov and Dicks (2004)
	Lactobacillus plantarum C11	Plantaricin EF, plantaricin JK and inductor factor plantaricin A	Two two-peptide with data not shown about molecular mass	Chromosomally encoded	*Lactobacillus* sp., *Pediococcus* sp., *Leuconostoc* sp. and *Streptococcus* sp.	Daeschel et al. (1990)
	L. plantarum LPC010	Plantaricin S and plantaricin T	Two-peptide, where plantaricin S had molecular mass about 2.5 kDa and the molecular mass of plantaricin T was not detected	Possible chromosomally encoded	*Propionibacterium* sp., *Clostridium tyrobutyricum* and *E. faecalis*	Díaz, Sánchez, Desmazeaud, Barba, and Piard (1993)
	L. plantarum ST16Pa	Bacteriocin ST16Pa	Single-peptide peptide with molecular mass estimated to be 6.5 kDa	Information about genetic determinates not shown	*E. faecalis*, *E. faecium*, *L. monocytogenes*, *Listeria innocua*, *S. aureus*, *Streptococcus* spp., and *Pseudomonas* spp.	Todorov et al. (2011)
	L. plantarum 163	Plantaricin 163	Single-peptide with molecular mass about 3.5 kDa	Information about genetic determinates not shown	*S. aureus*, *L. monocytogenes*, *B. pumilus*, *B. cereus*, *M. luteus*, *L. thermophilus*, *L. rhamnosus*, *E. coli*, *P. aeruginosa* and *P. fluorescens*	Hu et al. (2013)
Milk products	*L. plantarum* AMA-K	Bacteriocin AMA-K	Single-peptide with molecular mass about 2.9 kDa	Information about genetic determinates not shown	*Enterococcus* spp., *E. coli*, *Klebsiella pneumoniae* and *Listeria* spp.	Todorov, Nyati, Meincken, and Dicks (2007)
	L. plantarum WHE92	Pediocin AcH	Single-peptide with molecular mass about 4.5 kDa	Plasmid-encoded	*L. monocytogenes*	Ennahar et al. (1996)
	L. plantarum LB-B1	Pediocin LB-B1	Single-peptide with molecular mass estimated between 2.5 and 6.5 kDa	Plasmid-encoded	*Listeria* spp., *Lactobacillus* spp., *Streptococcus* spp., *Enterococcus* spp., *Pediococcus* spp. and *E. coli*	Xie et al. (2011)
	L. plantarum ST8KF	BacST8KF	Single-peptide with molecular mass about 3.5 kDa	Information about genetic determinates not shown	*L. casei*, *Lactobacillus salivarus*, *Lactobacillus curvatus* and *L. innocua*	Powell et al., 2007
Cereals	*L. plantarum* ST13BR	Bacteriocin ST13BR	Single-peptide with molecular mass about 10.0 kDa	Information about genetic determinates not shown	*P. aeruginosa*, *E. faecalis*, *K. pneumonia* and *E. coli*	Todorov, Van Reenen, & Dicks, 2004
	L. plantarum ST194BZ	ST194BZ(α) and ST194BZ(β)	Two-peptide (α and β-peptide) with molecular mass estimated to be 3.3 and 14.0 kDa, respectively	Information about genetic determinates not shown	*E. faecalis*, *E. coli*, *Enterobacter cloacae* and *P. aeruginosa*	Todorov and Dicks (2005)
	L. plantarum 423	Plantaricin 423	Single-peptide with molecular mass about 3.5 kDa	Plasmid-encoded	*B. cereus*, *C. sporogenes*, *E. faecalis*, *Listeria* spp. and *Staphylococcus* spp.	Reenen et al. (1998)

- Meat:

Various bacteriocinogenic strains of *L. plantarum* have been isolated from sausages obtained from different manufacturers under distinct ripening times (Garriga, Hugas, Aymerich, & Monfort, 1993).

Enan et al. (1996) isolated an antimicrobial substance produced from *L. plantarum* UG1 obtained from dry sausage. This substance was capable of inhibiting other strains of *Lactobacillus* and *Lactococcus* and some pathogenic strains, such as *L. monocytogenes*, *B. cereus*, *C. perfringens* and *C. sporogenes*. This antimicrobial compound was characterized as a bacteriocin and named plantaricin UG1. This is a single-peptide with a molecular mass between 3.0 and 10.0 kDa and its production appeared to be chromosomally encoded. In addition, a

LAB isolated from Italian sausages produced a bacteriocin, called plantaricin 35d (MW 4.5 kDa), having high antimicrobial activity against food pathogens (*S. aureus*, *L. monocytogenes* and *A. hydrophila*) (Messi et al., 2001).

The sausage has been employed as a continuous source of bacteriocinogenic cultures. For instance, Kanatani & Oshimura (1994) reported the production of a plasmid encoded bacteriocin called plantacin 154 with a molecular mass about 3.0 kDa or less, produced by *L. plantarum* LT154 strain isolated from dry sausage. Meanwhile Rekhif, Atrih, & Lefebvre (1995) obtained plantaricin SA6 from *L. plantarum* SA6, an isolate from fermented sausage. This is a single peptide with molecular mass of about 3.4 kDa and information about genetic determinants was not mentioned. A study by Todorov, Ho, Vaz-Velho, & Dicks (2010) characterized bacteriocins produced by the *L. plantarum* ST202Ch and ST216Ch strains isolated from beloura or chorizo, a traditional Portuguese product made of pork meat. The chromosomally encoded single peptide bacteriocins were denominated by bacST202Ch and bacST216Ch with molecular masses estimated to be 3.5 and 10 kDa, respectively, and were able to inhibit the growth of various Gram-positive and Gram-negative microorganisms considered deteriorative of meat products. The genes encoding bacteriocin ST202Ch were found identical to that reported for the structural gene encoding pediocin PA-1 (*pedA*, *pedB*, *pedC*, and *pedD*).

- Fish:

Noonpakdee et al. (2009) isolated the *L. plantarum* PMU 33 strain from som-fak, a Thai product made of fish with low salt contents. The bacteriocin purified and characterized from the culture supernatant consisted of two peptides with the molecular masses of 3.2 and 3.0 kDa. The molecular mass of this two-peptide bacteriocin was nearly identical to that of two-peptide plantaricin W (Plw) which consists of two peptides Plwα and Plwβ. The genes encoding these two peptides amplified by PCR with Plw gene specific primer showed identical sequences to Plwα and Plwβ. This bacteriocin was able to inhibit a large number of Gram-positive microorganisms considered pathogens and food spoilage microorganisms, such as *L. monocytogenes*, *B. cereus*, *E. faecalis* and *S. aureus*.

Fricourt, Barefoot, Testin, & Hayasaka (1994) isolated the *L. plantarum* BF001 strain from the flesh of processed and refrigerated catfish. This strain was able to produce an antimicrobial substance designated plantaricin F, a single-peptide with a molecular mass between 0.4 and 6.7 kDa. It was active against some bacteria of the genus *Lactobacillus*, *Lactococcus*, *Listeria*, *Micrococcus*, *Leuconostoc*, *Pediococcus*, *Staphylococcus*, *Streptococcus*, *Salmonella* and *Pseudomonas*.

- Fruits and vegetables:

Two bacteriocins, ST28MS and ST26MS, produced by different *L. plantarum* strains, were isolated from molasses and partially characterized. Both bacteriocins with molecular masses estimated to be 5.5 and 2.8 kDa, respectively, showed unusual antimicrobial activity against Gram-negative bacteria, including *P. aeruginosa*, *E. coli* and *A. baumanii*. No plasmids were recorded for strains ST28MS and ST26MS, suggesting that these bacteriocins are chromosomally encoded (Todorov & Dicks, 2004).

L. plantarum C-11 isolated from fermented cucumbers (Daeschel, McKenney, & McDonald, 1990) produced bacteriocins such as plantaricin EF and plantaricin JK (Anderssen et al., 1998). Plantaricin A, which was previously incorrectly identified as the bacteriocin responsible for the antimicrobial activity of *L. plantarum* C11, induces the production of the bacteriocins mentioned above (Meyer, Larsen, Sletten, Daeschel, & Nes, 1993).

Díaz et al. (1993) identified two bacteriocins produced by *L. plantarum* LPC010 isolated from fermented green olives. These antimicrobial substances were named plantaricin S (2.5 kDa), which is produced during the logarithmic growth phase, and plantaricin T (molecular mass not determined), produced when the microorganism reaches the stationary growth phase. The genetic determinants for plantaricin S and T production do not appear to be plasmid encoded.

Todorov et al. (2011) isolated the *L. plantarum* ST16Pa strain from papaya (*Carica papaya*). The bacteriocin produced by this strain, named ST16Pa (6.5 kDa), showed activity against different species of the genus *Enterobacter*, *Enterococcus*, *Lactobacillus*, *Pseudomonas*, *Streptococcus*, *Staphylococcus*, and some genres of *Listeria* spp. No information was given about genetic determinants.

Plantaricin 163 is a novel bacteriocin produced by *L. plantarum* 163 isolated from traditional Chinese fermented vegetables by Hu et al. (2013). This bacteriocin showed a broad-spectrum inhibitory activity not only against LAB but also against other Gram-positive and Gram-negative bacteria including *S. aureus*, *L. monocytogenes*, *B. pumilus*, *B. cereus*, *M. luteus*, *L. thermophilus*, *L. rhamnosus*, *E. coli*, *P. aeruginosa*, and *P. fluorescens*. The physicochemical studies of this bacteriocin (3.5 kDa) are in agreement with the characteristic features of antimicrobial peptides, thus indicating the potential value of plantaricin 163 as a biopreservative in the food industry (Hu et al., 2013). No information was given about genetic determinants.

Other bacteriocins are plantaricin C19 produced by *L. plantarum* C19 isolated from fermented cucumbers (Atrih, Rekhif, Moir, Lebrihi, & Lefebvre, 2001); plantaricin NA, produced by *L. plantarum* sp. isolated from vegetable origin (Olasupo, 1998); plantaricin-149, produced by *L. plantarum* NRIC 149 isolated from pineapple (Kato et al., 1994); and plantaricin D produced by *L. plantarum* BFE 905 isolated from "Waldorf" salad (Franz, Du Toit, Olasupo, Schillinger, & Holzapfel, 1998).

- Milk-based products:

Todorov, Nyati, Meincken, & Dicks (2007) reported the production of bacteriocin AMA-K by the *L. plantarum* AMA-K strain isolated from amasi, a product traditionally made of fermented milk that is consumed in different regions of Southern Africa, including Zimbabwe, South Africa and Lesotho. The bacteriocin AMA-K (2.9 kDa) inhibited the growth of *Enterococcus* spp., *E. coli*, *Klebsiella pneumoniae* and *Listeria* spp. No information was given about genetic determinants.

González, Arca, Mayo, & Suárez (1994) reported the production of plantaricin C by the *L. plantarum* LL441 strain isolated from Cabrales cheese. The bacteriocin showed bactericidal activity, followed by, in some cases, cell lysis.

Xie et al. (2011) demonstrated the presence of pediocin LB-B1 (single-peptide with molecular mass estimated between 2.5 and 6.5 kDa), which was produced by *L. plantarum* LB-B1 isolated from koumiss, a traditional Chinese fermented dairy product. In particular, this bacteriocin was active against *Listeria*, *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Pediococcus* and *E. coli* strains. The genetic determinants for pediocin LB-B1 production appeared to be plasmid-encoded.

Powell, Witthuhn, Todorov, & Dicks (2007) isolated *L. plantarum* ST8KF from kefir, a carbonated refreshing drink, and its bacteriocin (single-peptide with molecular mass about 3.5 kDa) showed activity against different microorganisms including *L. casei*, *L. salivarius*, *Lactobacillus curvatus* and *L. innocua*. No information was given about genetic determinants.

Other bacteriocins include plantaricin MG (molecular mass about 2.1 kDa) produced by *L. plantarum* KLDS1.0391 and isolated from "Jiaoke", a traditional fermented cream from China by Gong et al. (2010), being active against Gram-positive and Gram-negative bacteria including *L. monocytogenes*, *S. aureus*, *S. typhimurium* and *E. coli* and plantaricin LC74 (single-peptide with molecular mass about 5 kDa), produced by *L. plantarum* LC74 isolated from crude goat's milk. This bacteriocin showed a narrow spectrum of activity against several strains of mesophilic lactobacilli including *L. plantarum*, *Lactobacillus brevis* and *Lactobacillus büchneri* (Rekhif, Atrih, & Lefebvre, 1994).

- Cereals:

Todorov, Van Reenen, & Dicks (2004) reported the production of the bacteriocin ST13BR (single-peptide with molecular mass about

10 kDa) by the *L. plantarum* ST13BR strain isolated from barley beer, a traditional drink made of fermented corn, barley, soy flour and sugar, produced in South Africa. This bacteriocin was effective against *L. casei*, *P. aeruginosa*, *E. faecalis*, *K. pneumoniae* and *E. coli*. No information was given about genetic determinants. Todorov & Dicks (2005) described the isolation of the *L. plantarum* ST194BZ strain from boza, a fermented beverage typically from the eastern Balkan countries. It is one of the most traditional drinks produced by the fermentation of different cereals with the use of yeast and LAB. The ST194BZ strain was able to produce two types of bacteriocins, termed ST194BZα (3.3 KDa) and ST194BZβ (14 kDa), being active against a broad range of pathogens and spoilage bacteria including *E. faecalis*, *E. coli*, *Enterobacter cloacae* and *P. aeruginosa*. No information was given about genetic determinants.

Reenen et al. (1998) isolated the *L. plantarum* 423 strain from sorghum beer. This strain produced the bacteriocin plantaricin 423 (single-peptide with molecular mass about 3.5 kDa), which is capable of inhibiting a wide range of microorganisms, such as *B. cereus*, *C. sporogenes*, *E. faecalis*, *Listeria* spp. and *Staphylococcus* spp. DNA hybridization studies have shown homology between the plasmid DNA of *L. plantarum* 423 and the pediocin PA-1 operon. This suggests that plantaricin 423 is plasmid-encoded.

Additionally, the production of other bacteriocins has been described, including plantaricin K, produced by *L. plantarum* DK9 isolated from "fufu", a fermented cassava product (Olukoya, Tichaczek, Butsch, Vogel, & Hammes, 1993); plantaricin ST31, produced by *L. plantarum* ST31 isolated from sourdough (Todorov et al., 1999) and plantaricin KW30, produced by *L. plantarum* strain KW30 isolated from fermented corn (Kelly, Asmundson, & Huang, 1996).

4.3. Applications of L. plantarum and its bacteriocins

A wide variety of benefits associated with the use of *L. plantarum* as a potential probiotic has been reported in literature (Ningegowda & Gurudutt, 2012; Zago et al., 2011). In general, these studies reported that this LAB has been used to enhance intestinal barrier function and improve symptoms of irritable bowel syndrome (Anderson, Cookson, MacNabb, Kelly, & Roy, 2010; Ducrotté, Sawant, & Jayanthi, 2012). According to Axling et al. (2012), the use of *L. plantarum* can affect gut microbiota, lipid metabolism and inflammation in high-fat fed mice. Another example of health benefits was reported by Nguyen, Kang, & Lee (2007). In this study, *L. plantarum* PH04 was effective in cholesterol-lowering activities. On the other hand, Gallego et al. (2011) reported that the consumption of probiotic *L. plantarum* improves the general health status in older people.

Several substances have been produced by different *L. plantarum* strains isolated from different food sources. These strains of bacteriocinogenic *L. plantarum* are naturally present in these products, contributing to the improvement of organoleptic characteristics and playing an essential role in their biopreservation (Todorov, 2009).

Biopreservation is a technique used to prolong food shelf life and improve safety through the use of protective microbiota and/or its antimicrobial peptides, such as bacteriocins (Schillinger et al., 1996). The application of bacteriocins can help to reduce the use of chemical preservatives and/or the intensity of heat treatment, as well as prevent other physical treatments, satisfying the demand for "fresh", "ready-to-eat" foods with few preservatives (Todorov, 2009).

The bacteriocins produced by *L. plantarum* or even the strain itself are used in different situations. Penteado et al. (2007) conducted a study by inoculating bacteriocins from *L. plantarum* in silage of mombaça grass, with the purpose of improving the fermentation profile according to the values of pH, NH_3, lactic acid and acetic acid, favoring the development of LAB and lowering dry matter losses.

Campagnol, Fries, Terra, Santos, & Furtado (2007) produced a starter culture with a *L. plantarum* strain in the culture medium of porcine plasma and verified the feasibility of its application in salamis. As a result, salamis made with a *L. plantarum* starter culture promoted a higher microbiological safety to salamis, as well as a significant improvement of their flavor.

In the therapeutic field, the topical application of lactobacilli is receiving attention due to the prevention of superficial skin and burn wound infections. Brachkova et al. (2011) studied the properties of *L. plantarum* immobilized with calcium alginate films and investigated the antibacterial activity of these films in a model burn wound in rats. The study showed a significant decrease in the number of colonies of *P. aeruginosa* (one of the most frequently isolated pathogens in chronic infections, which was used to test the activity of *L. plantarum*), suggesting that the immobilization of *L. plantarum* by calcium alginate films may be a possible intervention for the prevention of infections caused by burns. Also in this context, Ramos et al. (2012) investigated the effects of *L. plantarum* supernatants on pathogenic properties of *P. aeruginosa*, such as adhesion, viability, virulence factors, biofilm formation, and quorum sensing signal expression. In this study, *L. plantarum* supernatants were able to inhibit pathogenic properties of *P. aeruginosa* by a quorum quenching mechanism. Also, the antipathogenic properties, along with the immunomodulatory, tissue repair, and angiogenesis properties in the *L. plantarum* supernatants, make them an attractive option in infected chronic wound treatment.

O'Shea et al. (2012) reported the problem related to the odor of pig manure. Conventional dietary strategies to reduce this odor can be costly, prevent nutrient digestibility, and receive varying responses. Alternatively, the authors proposed the use of *L. plantarum* in the diet of pigs, with or without supplementation of inulin, in order to reduce the manure odor without compromising the nutrient digestibility. It was found that dietary supplementation of pigs with *L. plantarum*, with or without inulin, reduces the manure odor.

4.4. Purification of bacteriocins produced by L. plantarum

Different strategies for the extraction of bacteriocins produced by *L. plantarum* from cultivation broths, and further purification to final products have already been described in literature (Atrih et al., 2001; Gong et al., 2010; Hata, Tanaka, & Ohmomo, 2010; Müller, Carrasco, Tonarelli, & Simonetta, 2009; Smaoui et al., 2010; Todorov, Velho, & Gibbs, 2004; Zhu, Zhao, Sun, & Gu, 2014). Purification methods, including salinization, solvent extraction, ultrafiltration, adsorption–desorption, ion exchange chromatography and high-performance liquid chromatography (HPLC), are the most common techniques (Parada et al., 2007). Other alternative purification methods include salting-out, gel filtration, or reverse-phase high-performance liquid chromatography (RP-HPLC) (Hu et al., 2013).

Some purification strategies with the respective specific activity and purification folds are shown in Table 2. All of them led to high levels of purification indicating their high degree of effectiveness.

The literature related to the purification of bacteriocins is vast, encompassing several types of protocols. However, all protocols involve the use of the HPLC technique as the final purification step (Hata et al., 2010; Smaoui et al., 2010; Zhu et al., 2014). Some authors employed the RP-HPLC technique, a variant of HPLC in which a hydrophobic stationary phase is employed, favoring, of course, the elution of polar molecules (Atrih et al., 2001; Gong et al., 2010; Müller et al., 2009). It must be borne out that by using the RP-HPLC technique as the final step, less purification procedures are required, thus decreasing the overall cost of the purification process.

Recently, a new procedure was proposed for the purification of macromolecules, including bacteriocins, based on the liquid–liquid extraction for aqueous two-phase micellar systems (ATPMS). This method can be applied for extracting bacteriocins directly from the fermented medium, leading to a simplification on the overall

Table 2
Strategies of purification to obtain bacteriocins produced by *Lactobacillus plantarum*.

Bacteriocin	Purification steps	Specific activity (AU/mg)	Purification (fold)	References
Plantaricin ZJ008	Culture supernatant	14.9	1.0	Zhu et al. (2014)
	Macroporous resin column	37.5	2.5	
	Cation exchenge	369.9	24.8	
	Chromatography	838.7	56.2	
	Gel filtration	8556.7	573.1	
	HPLC			
Plantaricin from *L. plantarum* LP31	Culture supernatant	85.5	1	Müller et al. (2009)
	Sep-Pack cartridges (C18)	5959	69.6	
	Gel-filtration chromatography	5900	689.5	
	RP-HPLC	506,000	5914.6	
Plantaricin C19	Culture supernatant	455	1	Atrih et al. (2001)
	Release of adsorbed bacteriocin from producing cells	17,808	39.1	
	Reverse phase high-performance liquid chromatography (HPLC)	409,600	900.2	
Plantaricin MG	Culture supernatant	0.37	1	Gong et al. (2010)
	Ammonium sulfate precipitation	5.35	14.0	
	Gel filtration	44.64	20.0	
	Reverse-phase chromatography	9333.33	25.2	
BacTN635	Culture supernatant	2083	1	Smaoui et al. (2010)
	Ammonium sulfate precipitation	9904	4.7	
	Centrifugal microconcentrators	14310	6.8	
	Gel filtration	146104	70.1	
	4 – HPLC	197,368	94.7	
Plantaricin ASM1	Culture supernatant	253	1	Hata et al. (2010)
	Ammonium sulfate precipitation	1850	7.3	
	Cation exchange	11,900	47.0	
	Chromatography	20,700	81.8	
	Octyl-Sepharose CL-4B column	10,700	42.3	
	HPLC			

purification protocol of bacteriocins (Jozala, Lopes, Novaes, Mazzola, & Pessoa-Jr, 2012; Liu, Nikas, & Blankschtein, 1996; Molino et al., 2014).

5. Conclusion

Nowadays, consumers tend to seek fresh and natural products, avoiding processed products containing chemical additives. Thus, many researchers began to look for natural and effective preservatives. The use of bacteriocins seems to be a great alternative, as they present activity against a wide range of food-borne pathogens and spoilage microorganisms.

Several substances have been isolated from various *L. plantarum* strains found in different niches, such as meat, fish, dairy products, fermented vegetables, cereals and fruits. Their application can be performed successfully in food-related fermentations, ensuring not only the organoleptic characteristics, but also contributing to increase the shelf life and safety of the final product.

Although there are many studies on the production of bacteriocins by *L. plantarum*, until this moment, there are no reports in literature describing the existence of a bacteriocin from *L. plantarum*, such as nisin and pediocin, available in the market. This fact could be due to the lack of an efficient or a low-cost purification strategy, which allows the bacteriocin produced by this microorganism to be commercially available. Therefore, future studies might be directed at the development of efficient and low-cost purification protocols.

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APÊNDICE 7. Overview of Lactobacillus plantarum as a promising bacteriocin producer among lactic acid bacteria

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APÊNDICE 7. Overview of Lactobacillus plantarum as a promising bacteriocin producer among lactic acid bacteria

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Original article

Effect of inulin on growth and bacteriocin production by *Lactobacillus plantarum* in stationary and shaken cultures

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Summary The prebiotic effect of inulin added to MRS medium on growth and bacteriocin production by *L. plantarum* ST16 Pa was investigated in stationary cultures in anaerobic jars with medium containing 0.025% sodium thioglycolate or in flasks shaken at 100 rpm. In the presence of 1% inulin in anaerobic stationary cultures, this strain produced lactic acid at a level that was 36.5% higher than in the absence of the polysaccharide. In shaken cultures without inulin, cell count was 54% higher than in the stationary ones. Under stationary conditions in anaerobic jars, the addition of inulin increased the maximum specific growth rate from 0.37 to 0.49 h^{-1} and reduced the generation time from 1.85 h to 1.40 h. Consequently, the exponential phase was shortened from 12 to 9 h when the cells were grown in stationary cultures with the oxygen scavenger. Despite this effect of inulin on growth rate, stationary cultures without inulin displayed higher antimicrobial activity against *Listeria monocytogenes* L104 (3200 AU/mL) than cultures with inulin (1600 AU/mL); therefore, inulin behaved as a compound able to accelerate growth rather than to stimulate bacteriocin production. The results presented in this study are very promising, as *L. monocytogenes* is a well-known foodborne pathogenic microorganism. Moreover, *L. plantarum* ST16 Pa has proven to be a potential producer of a natural food preservative at an industrial level.

Keywords Bacteriocin, inulin, *Lactobacillus plantarum*, probiotic, shaken culture, stationary culture, supplementation.

Introduction

Consumers have become more concerned about the amount of chemical additives in their diets and are seeking 'natural'" or chemical-preservative-free foods. Lactic acid bacteria (LAB) are widely used in the food industry to provide preservative functions, because of their ability to produce various antibacterial compounds, including bacteriocins (De Vuyst & Leroy, 2007). Bacteriocins are ribosomally synthesised antimicrobial peptides produced by bacteria that are active against other bacteria, either belonging to the same species or even across genera (Martinez *et al.*, 2013).

Lactobacillus plantarum, a generally recognised as safe (GRAS) microorganism, is a LAB that has been widely used for food processing (De Vuyst & Leroy, 2007; Barrangou *et al.*, 2012; Sabo *et al.*, 2014). Several *Lactobacillus* spp. have well-documented probiotic properties and can grow either in the absence or in the presence of O_2, which makes them aerotolerant anaerobic microorganisms (Götz *et al.*, 1980). *L. plantarum* is a versatile species as it is present in the microbiota of starchy foods, cereals, meat, dairy products, vegetables, fruits, beverages and intestinal tract of mammals (Klerebezem *et al.*, 2003; Todorov & Franco, 2010).

Inulin is a prebiotic fermentable soluble fibre that cannot be digested by the enzymes of the human gastrointestinal tract; therefore, it enters the large intestine serving as substrate for intestinal beneficial bacteria, promoting their growth and improving their performance (Roberfroid, 2007). The stimulatory effect of this biopolymer on the growth of probiotic microorganisms can be explained by the increased level of fructose released by its partial hydrolysis and its subsequent assimilation through the glycolytic pathway (Oliveira *et al.*, 2012). As the production of bacteriocins is often associated with high cell concentrations (Parente & Ricciardi, 1999), supplementation of the

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culture media with additional carbon sources such as inulin might stimulate their production.

This study aimed at investigating *L. plantarum* ST16 Pa growth and bacteriocin production in shaken and stationary cultures with and without the addition of inulin to the MRS medium.

Materials and methods

Bacterial strains and cultivation

The *Lactobacillus plantarum* ST16 Pa strain used in this study was isolated from papaya (*Carica papaya*) and characterised as a bacteriocin producer and potential probiotic (Todorov *et al.*, 2011, 2012). The microorganisms used as indicators in preliminary screening tests on filtrate samples from cultures grown under different conditions (shaken or stationary culture, with or without 1% inulin) were *Listeria monocytogenes* (L101, L104, L106, L302, L409, L506, L607, L620, L711, L724) and *Enterococcus* sp. (13LC23, 13BA22, 11EN3, 11EN4, 11EN5, 1LB8) provided by the Department of Food, Faculty of Pharmaceutical Sciences, University of São Paulo (São Paulo, SP, Brazil), and the Department of Veterinary, Faculty of Veterinary Medicine, Federal University of Viçosa (Viçosa, MG, Brazil), respectively. Only one concentration of inulin (1%) was tested because a preliminary (unpublished) study, in which this prebiotic was tested at different concentrations (1, 2 and 4%), showed no statistically significant difference among results. Additional screening tests were performed exclusively on samples from stationary cultures without inulin using *Listeria innocua* 2061, 2864, 2871, 2865, 2860, 2712, 2711, 2863, 2193, 2873 and *Lactobacillus sakei* ATCC 15521, all provided by FioCruz (Rio de Janeiro, RJ, Brazil). BHI or MRS broth (DIFCO, Detroit, MI, USA) was used for storage and cultivation of *Listeria* strains or of the other strains, respectively. All microorganisms were stored at −80 °C in the presence of 40% (v/v) glycerol.

To cultivate the indicator strains, 100 µL of stock cultures were added to 5 mL of medium and incubated at 100 rpm, 30 ± 0.5 °C for 18 h.

To prepare the *L. plantarum* ST16 Pa inoculum, 1 mL of stock culture was inoculated into Erlenmeyer flasks containing 100 mL of MRS broth. The flasks were placed on a rotatory shaker at 100 rpm, 30 ± 0.5 °C for 12 h.

Under shaken conditions, 10% (v/v) of inoculum was transferred to Erlenmeyer flasks containing 100 mL of MRS broth with or without 1% (w/v) inulin and incubated in a shaker at 100 rpm, 30 ± 0.5 °C for 48 h. For stationary conditions, 10% (v/v) of inoculum was transferred into flasks containing 9 mL of MRS broth with or without 1% (w/v) inulin and incubated in jar without agitation. A GasPak EZ Container System (BD Diagnostic Systems, Hunt Valley, MD, USA) was used inside the anaerobic jar, and 0.025% sodium thioglycolate (oxygen reducer) was added to the broth to ensure hypoxia. Cultures were grown in triplicate, and samples were collected every 3 h during the first 15 h of fermentation and then every 6 h.

Growth, pH and biochemical measurements

Growth of *L. plantarum* ST16 Pa was measured by counting colony-forming units (CFU) using serial dilutions in saline (0.85% w/v). One hundred mL of each dilution was spread over the surface of plates containing MRS broth supplemented with 2% (w/w) agar. The plates were incubated for 48 h. Subsequently, the values of the colony counts were converted to decimal log.

The progressive acidification of culture medium was followed with a pH meter (pH300M Analyser, São Paulo, SP, Brazil).

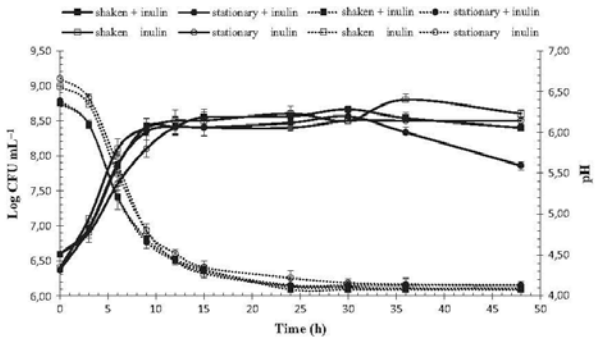

Figure 1 Growth curves of *Lactobacillus plantarum* ST16 Pa (continuous line) and pH values (pointed line).

All chemicals used in the experiments were of analytical reagent grade. High-purity glucose and lactic acid (Sigma-Aldrich, St. Louis, MO, USA) were used at concentrations from 0.1 to 2.0 g L^{-1} as standard solutions to prepare the calibration curve. In particular, the calibration curve was prepared by diluting the stock solutions with ultrapure water obtained from a Milli-Q system (Millipore, Bedford, MA, USA). The concentrations of glucose and lactic acid were determined by high performance liquid chromatography (HPLC). Briefly, each sample was centrifuged at 25 000 ***g*** for 10 min using a microcentrifuge (U-32R, Boeckel, Hamburg, Germany). The supernatant was filtered through a membrane with 0.22 μm pore diameter (Merck-Millipore, Darmstadt, Germany) and then injected into an HPLC system (Shimadzu, model Prominence, Kyoto, Japan), equipped with the following modules: two pumps (LC 20AD), column oven (CTO 20AC), degasser (model DGU-20A), an auto injector SIL-20ACHT adjusted to 30 μL volume injection, UV–vis SPD 20A, diode array SPDM 20A, with a working wavelength range of 190–230 nm, and refractive index (RID-10A) detectors, CBM controller 20A and Lab-Solutions software version 3.60. For the stationary phase, the Aminex HPX-87H ion exclusion column (300 × 7.8 mm, Bio-Rad Laboratories, USA) was used. Analyses were carried out at 50 °C, using 5.0 mM sulphuric acid as mobile phase at a flow rate of 0.6 mL min^{-1}, and a wavelength of 215 nm was selected for the quantification.

Table 1 Kinetic parameters of each culture conditions analysed

	Cultures conditions			
	Shaken	Shaken + 1% inulin	Stationary	Stationary + 1% inulin
Cell concentration (UFC mL^{-1})	5.9×10^8	5.3×10^8	3.2×10^8	3.5×10^8
μ_{max} (h^{-1})	0.53	0.48	0.37	0.49
t_g (h)	1.29	1.44	1.85	1.40

Bacteriocin bioassays

Culture samples were centrifuged at 20 000 ***g*** at 4 °C for 10 min. The pH of the supernatants was adjusted to 6.0–6.5 by addition of 1.0 M NaOH and heated to 80 °C for 10 min to inactivate proteases. Bacteriocin activity tests were performed according to the agar diffusion assay in Petri dishes containing 15 mL of BHI broth supplemented with 1% (w/w) agar and inoculated with an appropriate test microorganism. After solidification, 10 μL of cell-free supernatant were deposited over the plates, which were then incubated at 30 °C. Inhibition zones became visible after 18 to 24 h. Bacteriocin activity was quantified in triplicate by serial dilution of cell-free supernatant in 25 mM phosphate buffer at pH 6.5. The amount of bacteriocin produced by *L. plantarum* ST16 Pa was expressed in arbitrary units per mL (AU mL^{-1}) by the equation $AU/mL = D^n \times 1000/P$, where D = dilution factor, n = first dilution not exhibiting any inhibition zone, and P = volume of supernatant deposited onto the agar surface.

Statistical analysis

Biomass, pH, bacteriocin activity, glucose and lactic acid concentrations were submitted to analysis of variance (ANOVA) by the Statistica Software 12 (Tulsa, OK, USA).

Results and discussion

Acidification performance and cell growth kinetics

As illustrated in Fig. 1, *Lactobacillus plantarum* ST16 Pa was able to grow either in shaken or in stationary culture. Both modes of cultivations exhibited very simi-

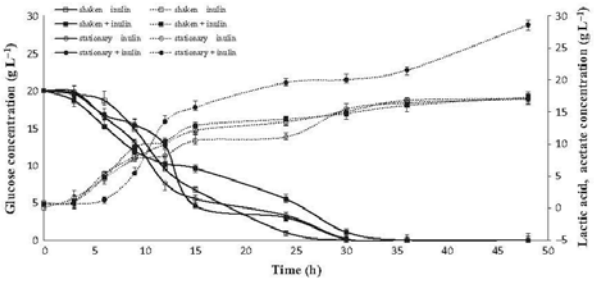

Figure 2 Concentrations of glucose (continuous line) and lactic acid (pointed line) during cultivations of *Lactobacillus plantarum* ST16 Pa.

lar acidification performances mainly associated with the release of lactic acid, with initial pH around 6.4–6.6 and final values of 4.1 in both cases. *L. plantarum* ST16 Pa stopped growing after approximately 24 h of cultivation, and the pH remained almost constant over the next 24 h. At this time, glucose was almost completely consumed. As suggested by Kouakou *et al.* (2010), such a low pH along with the presence of bacteriocin should be sufficient to prevent growth of foodborne pathogenic microorganisms such as *Listeria monocytogenes*.

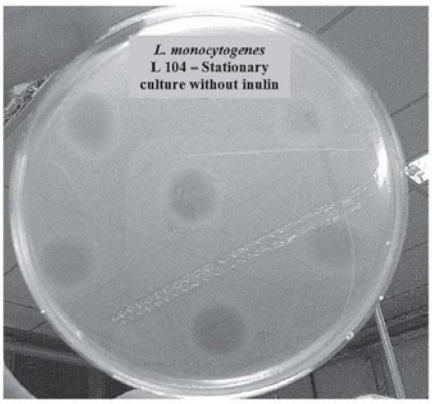

Figure 3 *In vitro* test of bacteriocin activity showing inhibition zones against *Lactobacillus monocytogenes* L104.

The maximum cell concentration ($X = 5.9 \times 10^8$ CFU mL^{-1}) in the shaken culture was more than 54% higher than that in the stationary one, the exponential growth phase was faster (9 h instead of 12 h), and the specific growth rate was significantly higher ($\mu_{max} = 0.53$ h^{-1} instead of 0.37 h^{-1}); consequently, the generation time was proportionally lower ($t_g = 1.29$ h vs. 1.85 h). These results show that the conditions adopted in shaken culture improved cell proliferation. Similar results were obtained by Gupta *et al.* (2011). Presumably, the agitation improved the distribution of nutrients, and the increased oxygen level in the medium could have led to higher yields of ATP per mol of consumed substrate (Fu & Mathews, 1999; Tortora *et al.*, 2012).

The shaken culture exhibited a slightly higher cell count ($X = 5.9 \times 10^8$ CFU mL^{-1}) than the stationary one ($X = 3.2 \times 10^8$ CFU mL^{-1}); therefore, 1% (w/w) inulin was added in the medium to stimulate growth. However, no statistically significant effect was observed on this parameter ($X = 5.3 \times 10^8$ and 3.5×10^8 CFU mL^{-1} for shaken and stationary cultures).

These results disagree with those of Huebner *et al.* (2007), who observed that supplementation of the culture medium with inulin led to a drastic decrease in cell concentration, depending on the strain of *L. plantarum*. Saulnier *et al.* (2007) claimed that inulin addition did not influence *L. plantarum* WCSF1 cell count, because the fructooligosaccharides released from inulin hydrolysis likely induced upregulation of genes involved in primary metabolism (fructokinases, phosphoenolpyruvate transport chain, β-fructofuranosidade, α-glucosidase) and regulated negatively genes for protein formation, cell wall and lipid production. A

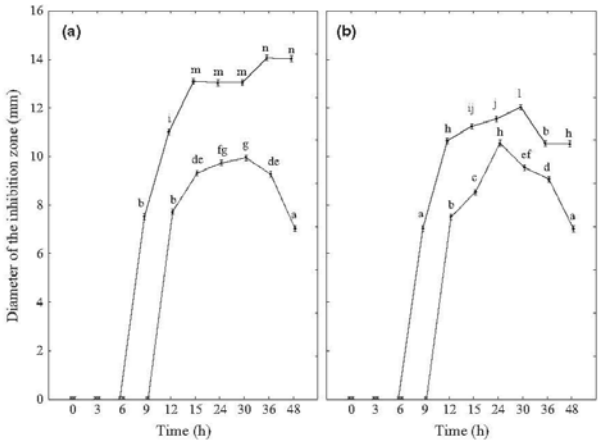

Figure 4 Inhibition zones against *Lactobacillus monocytogenes* L104 either in shaken (dotted line) or in stationary (continuous line) cultures, without (a) or with (b) 1% (w/w) inulin. Different letters mean statistically significant differences according to the test of Tukey ($\alpha < 0.05$)

Table 2 Results of bacteriocin assay against different target microorganisms either in stationary or in shaken flasks, with or without 1% (w/w) inulin

	Cultures conditions			
Indicator strains	Shaken	Shaken + 1% inulin	Stationary	Stationary + 1% inulin
Lactobacillus monocytogenes 106	–	–	–	–
L. monocytogenes 620	–	–	–	–
L. monocytogenes 409	–	–	–	–
L. monocytogenes 104	+	+	+++	++
L. monocytogenes 607	–	–	–	–
L. monocytogenes 302	–	–	–	–
L. monocytogenes 101	+	+	+++	++
L. monocytogenes 724	–	–	–	–
L. monocytogenes 711	+	+	+++	++
L. monocytogenes 506	–	–	–	–
Enterococcus sp. 13LC23	–	–	–	–
Enterococcus sp. 13BA22	–	–	–	–
Enterococcus sp. 11EN3	–	–	–	–
Enterococcus sp. 11EN4	–	–	–	–
Enterococcus sp. 11EN5	–	–	–	–
Enterococcus sp. 1LB8	–	–	–	–
L. innocua 2061	NT	NT	–	NT
L. innocua 2864	NT	NT	–	NT
L. innocua 2871	NT	NT	–	NT
L. innocua 2865	NT	NT	–	NT
L. innocua 2860	NT	NT	–	NT
L. innocua 2712	NT	NT	–	NT
L. innocua 2711	NT	NT	+++	NT
L. innocua 2863	NT	NT	–	NT
L. innocua 2193	NT	NT	–	NT
L. innocua 2873	NT	NT	+++	NT
L. innocua 2052	NT	NT	+++	NT
L. sakei ATCC 15521	NT	NT	+++	NT

NT, not tested.
Legend: 0–2 mm (–); 2–9 mm (+); 9–11 mm (++); 11–14 mm (+++).

similar occurrence may have taken place with the strain employed in this study.

On the other hand, the values of μ_{max} and t_g were appreciably influenced by the addition of inulin. As expected, the highest value of μ_{max} (0.53 h^{-1}) and lowest value of t_g (1.29 h) were obtained in shaken culture, but in the presence of 1% (w/w) inulin, the former parameter decreased to 0.48 h^{-1} and the latter increased by about 10%. Under stationary conditions, these variations were opposite and even more significant, in that μ_{max} increased from 0.37 to 0.49 h^{-1}, t_g decreased from 1.85 to 1.40 h, and consequently the exponential phase was shortened from 12 to 9 h. The values of cell concentration, μ_{max} and t_g of each culture condition were summarised in Table 1. Nagpal & Kaur (2011) obtained qualitatively similar results using various strains of *L. plantarum* under comparable conditions, even though no cell count was reported.

Glucose consumption and lactic acid release

As shown in Fig. 2, both stationary and shaken cultures released great amounts of lactic acid, but when stationary cultures were supplemented with 1% (w/w) inulin, the polysaccharide stimulated the production of lactic acid, whose concentration was 36.5% higher than that in the absence of inulin. Glucose concentration was about the same under all culture conditions (ca. 20 g L^{-1}); however, in the presence of inulin, it is likely that some fructose was available in the culture medium as a result of partial hydrolysis of the polysaccharide. In this case, presumably, fructose monomers released from inulin were assimilated by the EMP pathway, leading to higher concentrations of lactic acid under stationary conditions. A similar behaviour was observed with *L. plantarum* WCFS1, which produced higher lactic acid concentrations in anaerobic culture when supplemented with oligofructose from inulin hydrolysis (Saulnier *et al.*, 2007).

Bacteriocin activity

Of the 16 indicator strains used in preliminary screening tests carried out under different conditions (shaken or stationary culture, with or without 1% w/w inulin), only three showed significant inhibition zones induced by the released bacteriocin, namely *L. monocytogenes* L101, L104 and L711 (See Fig. 3 as an example). The results of these tests, which are illustrated in Fig. 4 in terms of mean inhibition zone versus time, show that the stationary culture without inulin provided the highest bacteriocin production and that the bacteriocin displayed a relatively narrow inhibition spectrum. Therefore, twelve additional target microorganisms were tested exclusively with culture supernatants from stationary culture without inulin. The results of these tests, summarised in Table 2 together with those of preliminary tests, show that *Listeria innocua* 2052, 2873, 2711 and *Lactobacillus sakei* ATCC 15521 were sensitive to the bacterio-

cin and suggest that its action spectrum may be wider than evidenced by preliminary experiments. In fact, 25% of the target microorganisms were shown to be sensitive to the bacteriocin, compared with 19% in the preliminary experiments.

The supernatant from stationary cultures without inulin showed the largest inhibition zones, corresponding to a bacteriocin activity of 1600 AU mL^{-1} against *L. monocytogenes* L101 and L711, *L. innocua* 2873, 2711 and 2052, and *L. sakei* ATCC 15521, as well as an activity of 3200 AU mL^{-1} against *L. monocytogenes* L104. However, when the last culture was supplemented with 1% (w/w) inulin, it showed a considerably smaller diameter of the inhibition zone and a reduction in the bacteriocin activity against *L. monocytogenes* L104 to 1600 AU mL^{-1} (Table 3). Gomes *et al.* (2012) reported similar results for sakacin 1, in that inulin reduced the bacteriocin activity from 6400 to 3200 AU mL^{-1}. Similarly, Todorov *et al.* (2011) observed that *L. plantarum* ST16 Pa produced less bacteriocin when the culture medium was supplemented with fructose, that is the monosaccharide released from inulin hydrolysis. Moreover, Todorov & Dicks (2004) reported that bacteriocin production is strongly dependent on the type of nutrient source provided in the culture medium and that its activity is not always related to cell mass or growth rate of the producer strain. In shaken flasks, the bacteriocin activity against the seven sensitive indicator strains (*L. monocytogenes* L101, L104, L711, *L. innocua* 2052, 2711, 2873, *L. sakei* ATCC 15221) was 1600 AU mL^{-1} regardless to the presence or the absence of inulin.

Table 3 Quantification of bacteriocin activity against the indicator strains

	Cultures conditions			
	Shaken	Shaken + 1% inulin	Stationary	Stationary + 1% inulin
Indicator strains	**Quantification of bacteriocin activity (AU mL^{-1})**			
Lactobacillus monocytogenes 104	1600	1600	3200	1600
L. monocytogenes 101	1600	1600	1600	1600
L. monocytogenes 711	1600	1600	1600	1600
L. innocua 2711	NT	NT	1600	NT
L. innocua 2873	NT	NT	1600	NT
L. innocua 2052	NT	NT	1600	NT
L. sakei ATCC 15521	NT	NT	1600	NT

NT, not tested.

Conclusions

The results of shaken and stationary cultures of *Lactobacillus plantarum* ST16 Pa in MRS medium either with or without supplementation of 1% (w/w) inulin were compared. *L. plantarum* ST16 Pa was shown to effectively grow under both conditions, but the highest bacteriocin production (3200 AU/mL) was obtained in stationary culture without inulin. Inulin, while accelerating cell growth, was not appreciably metabolised as a carbon source for bacteriocin production, thus behaving as an actual prebiotic ingredient. The stationary culture without inulin showed inhibition zones against various strains of *Listeria monocytogenes* (L101, L104, L711) and *Listeria innocua* (2052, 2873, 2711) as well as against *Lactobacillus sakei* (ATCC 15521), among which *L. monocytogenes* L104 resulted to be the most sensitive one. Taking into account that *L. monocytogenes* is a well-known foodborne pathogenic microorganism, these results are quite promising for possible use of *L. plantarum* ST16 Pa as a producer of a natural food preservative at an industrial level. However, further studies are needed to produce the bacteriocin in bioreactors, that is under more controlled conditions, as well as to check its actual effectiveness as a preservative in foods. In addition, because foods are often subject to processes inducing stress factors to bacteriocinogenic bacteria, it would be essential, from an industrial viewpoint, to identify what stress conditions could substantially enhance bacteriocin production by *L. plantarum* ST16 Pa.

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Optimisation of cheese whey enzymatic hydrolysis and further continuous production of antimicrobial extracts by *Lactobacillus plantarum* CECT-221

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The enzymatic hydrolysis of cheese whey was optimised using the enzymes iZyme, Alcalase or Flavourzyme under different conditions. Hydrolysates supplemented with commercial nutrients were evaluated as fermentation broths to produce DL-3-Phenyllactic acid (PLA) from phenylalanine (Phe) by *Lactobacillus plantarum* CECT-221. Optimised hydrolysates were obtained using Flavourzyme at 50 °C and 100 rpm during 12 h, and assayed in 250 ml Erlenemyer flasks using different proportions of vinasses as economic nutrient. The process was then scaled up using a 2 litres Bioreactor working under the continuous modality. Under the intermediate dilution rate of 0·0207 h^{-1} 0·81 ± 0·026 mM of PLA and 38·8 ± 3·253 g/l of lactic acid were produced. A final evaluation revealed that lactic acid, and bacteriocins exerted the highest inhibitory effect among the extracted components of cell-free supernatants.

Keywords: Cheese whey, vinasses, antimicrobial extracts, *Lactobacillus plantarum*, phenyllactic acid, bacteriocins.

Biopreservation, the use of microorganisms and/or their metabolites to prevent spoilage and to extend the shelf life of foods, has growing interest due to the continuous request for food-grade chemical antifungal agents (Cortés-Zavaleta et al. 2014). The use of naturally occurring antimicrobials in foods retains the nutritive value of food without producing side effects (Kumar et al. 2013). In this context, lactic acid bacteria (LAB) produce a variety of antifungal substances such as organic acids, proteinaceous compounds and various low-molecular mass substances, including DL-3-Phenyllactic acid (PLA) (Yang & Chang, 2010), an organic acid by-product of phenylalanine metabolism that has been produced by several microorganisms, particularly using some strains of LAB (Valerio et al. 2004; Li et al. 2007; Prema et al. 2008; Mu et al. 2009; Zheng et al. 2011; Rodríguez et al. 2012; Rodríguez-Pazo et al. 2013). Due to its broad inhibitory activity against a variety of foodborne microorganisms, PLA has interesting potential for practical application as an antimicrobial agent in the food industry (Mu et al. 2009) providing new perspectives for the possibility of using this natural antimicrobial compound to control fungal contaminants and extend the shelf life of food and/or feedstuffs (Lavermicocca et al. 2003).

In spite of their wide application in the food industry, LAB are catalogued as fastidious-growing microorganisms with numerous requirements for growth including amino acids, peptides, vitamins, and nucleic acids (Brinques et al. 2010). Thus, although industry and consumers for food-related applications prefer products obtained by biotechnological procedures, fermentation technologies must be cost competitive with chemical synthesis to carry out the biotechnological process at an industrial scale (Bustos et al. 2004). Therefore, alternative low-cost media should be developed to efficiently compete with the costly synthetic media for large-scale commercial applications (Brinques et al. 2010). In this sense, cheese whey and vinification lees, two by-products of the dairy and wine industries, respectively, could be assayed to formulate economic fermentative media.

Considering that cheese whey is a carbohydrate reservoir of lactose and also contains essential nutrients, it can be

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employed as a cheap carbohydrate source for the biotechnological production of biomass or high-added extracellular products (Brinques et al. 2010; Panesar et al. 2010; Rodríguez-Pazo et al. 2013). On the other hand, vinasses, the main liquid wastes from the distillation process, are also alternative sources of financially competitive nutrients already efficiently employed for the economical production of food additives (Bustos et al. 2004; Salgado et al. 2009).

Among LAB, *Lactobacillus plantarum*, a heterofermentative metabolism with high acid tolerance and generally regarded as safe organism (GRAS), is one of the most widespread species used in food technologies. In this work, the production of PLA by *L. plantarum* was evaluated using cheese whey as an inexpensive source of carbon (lactose) and phenylalanine in batch cultures, after optimisation of the cheese whey enzymatic hydrolysis using three enzymes: iZyme, Flavourzyme and Alcalase. Vinasess were assayed as alternative economic nutrients to provide nitrogen compounds, mineral salts, and essential nutrients for microbial growth in batch or continuous fermentations. Finally, the antimicrobial activity of the cell-free supernatants was assayed against *Carnobacterium piscicola*.

Materials and methods

Materials

Cheese whey, kindly provided by the cheese plant Ruta Xacobea S.L. in Brea (A Coruña, Spain), and wine lees, kindly supplied by the winery Adegas San Roque (Beade, Ourense, Spain) were stored at 4 °C until use.

Cheese whey enzymatic hydrolysis

Flavourzyme (EC 3.4.11.1) with endo- and exoprotease activities, and Alcalase (EC 3.4.21.62) and iZyme BA (EC 3.4.21.1) with endoprotease activities, kindly provided by Novozymes (Copenhagen, Denmark), were used to carry out the enzymatic hydrolysis of the protein fraction of cheese whey in order to improve the substrate (phenylalanine) concentration and/or protein co-substrate that could stimulate the PLA production. The enzymatic hydrolysis was carried out for 1 or 12 h at 50 °C in a thermostatic bath, using the necessary amount (V) of enzyme to hydrolyse 10 g/l of protein. Three enzymes were assayed according to the following activities: flavourzyme with activity equivalent to 1000 Leucine aminopeptidase units (LAPU)/g; alcalase with activity equivalent to 2·4 Anson units (AU)/g, and iZyme BA with activity equivalent to 0·15 Anson units (AU)/g. Additional experiments were also conducted using 2 V. In all cases, cheese whey was initially adjusted to pH 7 with 5 N NaOH. Table 1 summarises all the experiments conducted.

Strains, growth conditions and inoculum preparation

Lactobacillus plantarum CECT-221, obtained from the Spanish Collection of Type Cultures (Valencia, Spain), was employed to assess its ability to produce different antimicrobial metabolites, meanwhile *Carnobacterium piscicola* CECT 4020 was employed as the target organism to evaluate the bacteriocin activity of commercial acids and cell-free supernatants (CFS) obtained after fermentation processes. Seeds of *L. plantarum* or *C. piscicola* were activated in 250 ml Erlenmeyer flasks using 100 ml of the Man-Rogosa-Sharpe (MRS) medium containing 10 g/l peptone, 8 g/l beef extract, 4 g/l yeast extract, 20 g/l D(+)-glucose, 2 g/l K_2HPO_4, 2 g/l diammonium hydrogen citrate, 5 g/l CH_3COONa, 0·2 g/l $MgSO_4 \cdot 7H_2O$, 0·05 g/l $MnSO_4 \cdot 2H_2O$ and 1 g/l Tween-80 at 31·5 °C and 100 rpm in orbital shakers according to the conditions reported by Rodríguez-Pazo et al. (2013). Aliquots of culture (0·5 ml) from the late log/stationary phase were dispensed into cryovials and maintained at −80 °C using 15% (v/v) glycerol as cryoprotectant.

Inocula to carry out fermentations were prepared by transferring one stock cryovial into 250 ml Erlenmeyer flasks containing 100 ml of activation medium. Seed activation cultures were grown for 12 h at 31·5 °C and 100 rpm in orbital shakers. At least two generations of activation cultures were required before inoculation. The cells from this culture were finally recovered by centrifugation at 2755 ***g*** for 15 min at 10 °C and used to inoculate fermentations with 5% (v/v) of the final fermentation volume. pH was initially corrected to 6·2 with 5 N NaOH and sterilised at 101 °C for 60 min in autoclave.

Fermentation of cheese whey

Raw cheese whey and all the hydrolysates obtained after enzymatic hydrolysis were assayed as fermentation media for the formation of PLA by *L. plantarum*. Cheese whey hydrolysates (100 ml) were supplemented with the nutrients of Brinques et al. (2010) containing 15 g/l peptone, 5 g/l yeast extract, 0·2 g/l $MgSO_4 \cdot 7H_2O$ and 0·04 g/l $MnSO_4 \cdot 2H_2O$, or remained without nutrients addition, to evaluate the optimal hydrolysis conditions.

Optimised hydrolysates were supplemented with vinasses. Before use, vinasses were centrifuged and the liquid fraction neutralised with NaOH to pH 7. The liquid fraction was frozen, and subsequently freeze-dried. The solid fraction was keep at refrigerator. Different volumes of freeze-dried vinasses (25, 50, 75, 100 ml) or 50 ml plus 20 g/l of solid fraction were resuspended in 100 ml final volume of previously hydrolysed cheese whey, to assess the possibility of use vinasses as only and economic nitrogen and mineral salts source for fermentation process.

All fermentations were carried out placing 250 ml Erlenmeyer flasks (containing 100 ml of culture medium) in orbital shakers at 31 °C and 100 rpm. Samples were withdrawn at different intervals to monitor fermentation for up to 80 h. Initially, 20 g/l of calcium carbonate was added to neutralise the acids produced.

Additional experiments were performed in continuous fermentation using a 2 l Braun Biostat fermenter at 31·5 °C

Table 1. Influence of nutrients addition and conditions of cheese whey enzymatic hydrolysis on fermentation parameters

Run	Enzyme	Time EH (h)	*V*	Nut.	Time of ferment. (h)	$Phe_{t=0}$ (mg/l)	$Phe_{(final)}$ (mg/l)	PLA (mM)	Q_{PLA} (mg/l·h)	$Y_{PLA/Phe}$ (g/g)
(a) F1	–	–	–	–	78	$4{\cdot}3 \pm 0{\cdot}283^{a}$	$104{\cdot}3 \pm 6{\cdot}505^{c}$	$0{\cdot}017 \pm 0{\cdot}006^{a}$	$0{\cdot}036 \pm 0{\cdot}012^{a}$	–
(a) F2	–	–	–	+	71	$152{\cdot}9 \pm 6{\cdot}364^{bc}$	$21{\cdot}4 \pm 5{\cdot}798^{a}$	$0{\cdot}93 \pm 0{\cdot}085^{fg}$	$2{\cdot}188 \pm 0{\cdot}199^{def}$	$1{\cdot}18 \pm 0{\cdot}102^{ab}$
(b) F3	Alc.	1	V	–	54	$11{\cdot}2 \pm 0{\cdot}283^{a}$	$25{\cdot}4 \pm 8{\cdot}485^{a}$	$0{\cdot}035 \pm 0{\cdot}008^{ab}$	$0{\cdot}108 \pm 0{\cdot}026^{a}$	–
(b) F4	Flav.	1	V	–	78	$102{\cdot}6 \pm 4{\cdot}243^{b}$	$7{\cdot}7 \pm 1{\cdot}980^{a}$	$0{\cdot}57 \pm 0{\cdot}099^{cde}$	$1{\cdot}215 \pm 0{\cdot}211^{bcd}$	$0{\cdot}99 \pm 0{\cdot}108^{a}$
(b) F5	Izy.	1	V	–	78	$5{\cdot}9 \pm 0{\cdot}566^{a}$	$6{\cdot}9 \pm 1{\cdot}556^{a}$	$0{\cdot}093 \pm 0{\cdot}008^{ab}$	$0{\cdot}198 \pm 0{\cdot}018^{ab}$	–
(b) F6	Alc.	1	V	+	46	$181{\cdot}8 \pm 4{\cdot}808^{c}$	$109{\cdot}4 \pm 16{\cdot}829^{c}$	$0{\cdot}77 \pm 0{\cdot}085^{efg}$	$2{\cdot}782 \pm 0{\cdot}307^{fg}$	$1{\cdot}78 \pm 0{\cdot}100^{ab}$
(b) F7	Flav.	1	V	+	46	$260{\cdot}7 \pm 28{\cdot}709^{d}$	$124{\cdot}0 \pm 12{\cdot}728^{c}$	$0{\cdot}64 \pm 0{\cdot}113^{def}$	$2{\cdot}312 \pm 0{\cdot}409^{ef}$	$0{\cdot}79 \pm 0{\cdot}103^{a}$
(b) F8	Izy.	1	V	+	54	$166{\cdot}4 \pm 7{\cdot}637^{c}$	$4{\cdot}1 \pm 2{\cdot}546^{a}$	$1{\cdot}02 \pm 0{\cdot}099^{gh}$	$3{\cdot}139 \pm 0{\cdot}305^{fgh}$	$1{\cdot}05 \pm 0{\cdot}134^{ab}$
(b) F9	Alc.	1	2 V	+	46	$182{\cdot}6 \pm 9{\cdot}899^{c}$	$115{\cdot}7 \pm 7{\cdot}495^{c}$	$0{\cdot}75 \pm 0{\cdot}099^{efg}$	$2{\cdot}710 \pm 0{\cdot}358^{fg}$	$1{\cdot}96 \pm 0{\cdot}756^{ab}$
(c) F10	Flav.	1	2 V	+	46	$278{\cdot}7 \pm 20{\cdot}930^{d}$	$287{\cdot}8 \pm 9{\cdot}899^{e}$	$0{\cdot}27 \pm 0{\cdot}099^{abc}$	$0{\cdot}976 \pm 0{\cdot}358^{abc}$	–
(c) F11	Izy.	1	2 V	+	46	$170{\cdot}4 \pm 8{\cdot}061^{c}$	$183{\cdot}7 \pm 2{\cdot}263^{d}$	$0{\cdot}35 \pm 0{\cdot}057^{bcd}$	$1{\cdot}265 \pm 0{\cdot}204^{cde}$	–
(c) F12	Alc.	12	V	+	46	$194{\cdot}9 \pm 4{\cdot}808^{c}$	$122{\cdot}5 \pm 5{\cdot}940^{c}$	$1{\cdot}04 \pm 0{\cdot}085^{gh}$	$3{\cdot}758 \pm 0{\cdot}307^{gh}$	$2{\cdot}39 \pm 0{\cdot}157^{b}$
(c) F13	Flav.	12	V	+	54	$272{\cdot}9 \pm 28{\cdot}991^{d}$	$159{\cdot}2 \pm 11{\cdot}455^{d}$	$1{\cdot}30 \pm 0{\cdot}071^{h}$	$4{\cdot}001 \pm 0{\cdot}218^{h}$	$2{\cdot}01 \pm 0{\cdot}611^{ab}$
(c) F14	Izy.	12	V	+	54	$188{\cdot}8 \pm 13{\cdot}435^{c}$	$66{\cdot}8 \pm 6{\cdot}364^{b}$	$1{\cdot}26 \pm 0{\cdot}113^{h}$	$3{\cdot}878 \pm 0{\cdot}348^{h}$	$1{\cdot}73 \pm 0{\cdot}126^{ab}$

Run	Enzyme	Time EH (h)	*V*	Nut.	$Lactose_{t=0}$(g/l)	$Lactose_{t=f}$(g/l)	$LA_{t=0}$(g/l)	$LA_{t=f}$(g/l)	Q_{LA} (g/l·h)	$Y_{LA/Lactose}$(g/g)	$AcH_{t=f}$(g/l)
(a) F1	–	–	–	–	$48{\cdot}5 \pm 1{\cdot}697^{cd}$	$48{\cdot}1 \pm 1{\cdot}273^{a}$	$0{\cdot}39 \pm 0{\cdot}042^{a}$	$0{\cdot}39 \pm 0{\cdot}085^{a}$	–	–	$0{\cdot}63 \pm 0{\cdot}023^{h}$
(a) F2	–	–	–	+	$48{\cdot}3 \pm 2{\cdot}546^{cd}$	0	$0{\cdot}39 \pm 0{\cdot}028^{a}$	$33{\cdot}5 \pm 0{\cdot}566^{c}$	$0{\cdot}467 \pm 0{\cdot}031^{ab}$	$0{\cdot}69 \pm 0{\cdot}085^{bcd}$	$4{\cdot}3 \pm 0{\cdot}042^{ae}$
(b) F3	Alc.	1	V	–	$49{\cdot}0 \pm 1{\cdot}131^{d}$	$47{\cdot}6 \pm 0{\cdot}849^{a}$	$0{\cdot}40 \pm 0{\cdot}014^{a}$	$1{\cdot}3 \pm 0{\cdot}283^{a}$	$0{\cdot}012 \pm 0{\cdot}003^{d}$	$1{\cdot}9 \pm 0{\cdot}410^{e}$	0
(b) F4	Flav.	1	V	–	$48{\cdot}7 \pm 1{\cdot}273^{d}$	$28{\cdot}0 \pm 1{\cdot}838^{b}$	$0{\cdot}47 \pm 0{\cdot}057^{a}$	$17{\cdot}0 \pm 1{\cdot}414^{b}$	$0{\cdot}232 \pm 0{\cdot}013^{e}$	$0{\cdot}80 \pm 0{\cdot}049^{cd}$	$2{\cdot}9 \pm 0{\cdot}157^{c}$
(b) F5	Izy.	1	V	–	$47{\cdot}9 \pm 2{\cdot}263^{cd}$	$47{\cdot}6 \pm 2{\cdot}121^{a}$	$0{\cdot}50 \pm 0{\cdot}014^{a}$	$0{\cdot}54 \pm 0{\cdot}099^{a}$	$0{\cdot}001 \pm 0{\cdot}000$	$0{\cdot}14 \pm 0{\cdot}004^{ab}$	0
(b) F6	Alc.	1	V	+	$47{\cdot}4 \pm 2{\cdot}121^{cd}$	0	$0{\cdot}44 \pm 0{\cdot}014^{a}$	$39{\cdot}6 \pm 1{\cdot}556^{de}$	$0{\cdot}551 \pm 0{\cdot}011^{ac}$	$0{\cdot}83 \pm 0{\cdot}021^{cd}$	$5{\cdot}0 \pm 0{\cdot}141^{ab}$
(b) F7	Flav.	1	V	+	$46{\cdot}4 \pm 2{\cdot}404^{cd}$	0	$0{\cdot}47 \pm 0{\cdot}071^{a}$	$39{\cdot}3 \pm 0{\cdot}990^{de}$	$0{\cdot}546 \pm 0{\cdot}017^{ac}$	$0{\cdot}84 \pm 0{\cdot}021^{d}$	$5{\cdot}0 \pm 0{\cdot}283^{ab}$
(b) F8	Izy.	1	V	+	$47{\cdot}1 \pm 1{\cdot}131^{cd}$	0	$0{\cdot}49 \pm 0{\cdot}028^{a}$	$41{\cdot}7 \pm 1{\cdot}980^{e}$	$0{\cdot}580 \pm 0{\cdot}023^{c}$	$0{\cdot}87 \pm 0{\cdot}007^{d}$	$4{\cdot}0 \pm 0{\cdot}141^{de}$
(b) F9	Alc.	1	2 V	+	$47{\cdot}3 \pm 0{\cdot}566^{cd}$	0	$0{\cdot}40 \pm 0{\cdot}099^{a}$	$38{\cdot}8 \pm 0{\cdot}566^{de}$	$0{\cdot}541 \pm 0{\cdot}013^{ac}$	$0{\cdot}81 \pm 0{\cdot}017^{cd}$	$5{\cdot}3 \pm 0{\cdot}170^{b}$
(c) F10	Flav.	1	2 V	+	$46{\cdot}8 \pm 0{\cdot}990^{cd}$	$47{\cdot}4 \pm 0{\cdot}14^{a}$	$0{\cdot}51 \pm 0{\cdot}028^{a}$	$0{\cdot}61 \pm 0{\cdot}170^{a}$	$0{\cdot}002 \pm 0{\cdot}001$	$0{\cdot}24 \pm 0{\cdot}321^{abc}$	$3{\cdot}4 \pm 0{\cdot}212^{cd}$
(c) F11	Izy.	1	2 V	+	$45{\cdot}7 \pm 2{\cdot}121^{bcd}$	$45{\cdot}1 \pm 1{\cdot}414^{a}$	$0{\cdot}49 \pm 0{\cdot}099^{a}$	$0{\cdot}51 \pm 0{\cdot}028^{a}$	$0{\cdot}001 \pm 0{\cdot}001$	$0{\cdot}03 \pm 0{\cdot}002^{a}$	$2{\cdot}0 \pm 0{\cdot}113^{i}$
(c) F12	Alc.	12	V	+	$41{\cdot}9 \pm 0{\cdot}566^{abc}$	0	$2{\cdot}9 \pm 0{\cdot}424^{b}$	$36{\cdot}0 \pm 1{\cdot}131^{cd}$	$0{\cdot}467 \pm 0{\cdot}013^{ab}$	$0{\cdot}79 \pm 0{\cdot}007^{cd}$	$6{\cdot}3 \pm 0{\cdot}170^{f}$
(c) F13	Flav.	12	V	+	$39{\cdot}0 \pm 1{\cdot}13^{a}$	0	$3{\cdot}5 \pm 0{\cdot}566^{b}$	$36{\cdot}7 \pm 1{\cdot}838^{cd}$	$0{\cdot}468 \pm 0{\cdot}001^{ab}$	$0{\cdot}85 \pm 0{\cdot}037^{d}$	$7{\cdot}3 \pm 0{\cdot}184^{g}$
(c) F14	Izy.	12	V	+	$39{\cdot}5 \pm 1{\cdot}697^{ab}$	0	$3{\cdot}4 \pm 0{\cdot}707^{b}$	$32{\cdot}5 \pm 1{\cdot}556^{c}$	$0{\cdot}410 \pm 0{\cdot}057^{b}$	$0{\cdot}74 \pm 0{\cdot}082^{cd}$	$6{\cdot}6 \pm 0{\cdot}283^{fg}$

(a) Influence of nutrients addition on crude cheese whey: FI and F2; (b) Influence of enzymatic hydrolysis of whey during 1 h and nutrients addition: F3 to F8; (c) Influence of the enzymatic hydrolysis conditions: F9 to F14.

Alc., Alcalase; Flav., Flavourzyme; Izy., Izyme; EH, enzymatic hydrolysis; Phe, phenylalanine; PLA, DL-3-Phenyllactic acid; LA, lactic acid; AcH, acetic acid. *V*: necessary amount of enzyme to hydrolyse 10 g/l of protein. Nut.: nutrients reported by Brusch Brinques et al. (2010); Q_{PLA}: global volumetric productivity of PLA; $Y_{PLA/Phe}$: Phe to PLA yield, calculated as $(PLA_{(final)}-PLA_{(t=0)})/(Phe_{t=0}-Phe_{(final)})$ without considering the amount of phenylalanine released in the course of fermentation; Q_{LA}: global volumetric productivity of LA; $Y_{LA/Lactose}$: Lactose to LA yield. Different letters mean statistically significant differences among values in the same column ($P<0{\cdot}05$).

and 100 rpm, with pH automatically controlled to 6·2 with 5 N NaOH as described by Rodríguez et al. (2012) and Rodríguez-Pazo et al. (2013). Fermentations were carried out working with 1800 ml corresponding: 1615 ml to sterilised cheese whey hydrolysates; 100 ml of nutrients elaborated after resuspending 50 ml freeze-dried and 20 g/l solid vinasses in 100 ml hydrolysed cheese whey; and 85 ml of seed culture used for inoculation.

All fermentations were performed in duplicate and standard deviation of mean values reported in the text.

Kinetic parameters and yields of fermentations

The kinetic parameters and yields of fermentations were calculated at the time where the PLA was maximal. Global volumetric productivity of PLA (Q_{PLA}, g/(L·h)) was calculated as the ratio between the PLA concentration (mg/l) and the fermentation time (h). Phe to PLA yield ($Y_{PLA/Phe}$ g/g) was defined as the ratio between PLA produced ($PLA_{final}-PLA_{t=0}$) and Phe consumed ($Phe_{t=0}-Phe_{final}$) without considering the amount of phenylalanine released in the course of fermentation. Global volumetric productivity of LA (Q_{LA}, g/(L·h)) was calculated as the ratio between the LA concentration (g/l) and the fermentation time (h). Lactose to LA yield ($Y_{LA/Lactose}$, g/g) was defined as the ratio between LA produced ($LA_{final}-LA_{t=0}$) and lactose consumed ($Lactose_{t=0}-Lactose_{final}$).

Antimicrobial effect of CFS

Cell-free supernatants (CFS) were obtained by continuous fermentation of cheese whey hydrolysates supplemented with vinasses using a dilution rate of 0·0207 h^{-1}. The hydrolysates were centrifuged at 2755 ***g*** for 15 min and 10 °C and filter-sterilised using 0·22 µM pore-size membranes to obtain CFS. The bacteriocin activity was determined according to the well-diffusion method described by Rodríguez-Pazo et al. (2013) against *C. piscicola*.

Analytical methods

Total solids in cheese whey were determined by dry weight using 5 g of sample maintained in the oven at 105 °C until constant weight. Total protein concentration was determined by the Pierce Bicinchoninic Acid assay Kit (Thermo Scientific). Fat content in whey (before or after enzymatic hydrolysis with Flavourzyme at 50 °C and 100 rpm during 12 h) was quantified according to ISO 1443. Metals and amino acids were quantified as described Salgado et al. (2009).

Samples were taken during fermentation and centrifuged at 2755 ***g*** for 15 min at 4 °C using a Centrifuge EBA 20. Supernatants were filtered by 0·22 µM pore-size membranes (Millipore) for lactose, lactic acid, acetic acid, Phe and PLA analyses by high-performance liquid chromatographic (HPLC) under the conditions described by Rodríguez-Pazo et al. (2013), while the cells were used for biomass concentration determination. Cells from a known volume of culture media were washed twice with distilled water and centrifuged under the same conditions reported previously. The resulting pellets were oven-dried at 105 °C to constant weight.

Statistical analysis

Mean values of fermentations were submitted to analysis of variance (ANOVA) by the Statistica Software 13.0. They were compared using the Tukey's test at significance level (P) < 0·05, and different letters were used to label values with statistically significant differences among them.

Results and discussion

Optimisation of cheese whey enzymatic hydrolysis

The production of DL-3-Phenyllactic acid (PLA) requires the availability of phenylalanine (Phe) among other compounds in the culture broth, considering that LAB have complex nutrient requirements since they have a limited capacity to synthesise vitamin B and amino acids (de Lima et al. 2010). Hence, the controlled hydrolysis of cheese whey proteins with specific enzymes, could be used to provide a nitrogen source suitable for growth promotion in industrial fermentation, thus eliminating, or reducing, the need for expensive supplements (Galvão et al. 2009).

Using the necessary amount of enzyme to hydrolyse 10 g/l of protein during 1 h (V), flavourzyme showed the higher amounts of free Phe (107·8 mg/l), followed by alcalase (28·9 mg/l) and iZyme (13·5 mg/l), that is at least 3-fold higher than untreated cheese whey content of Phe (4·3 mg/l). An increment in time from 1 to 12 h, increased the concentration of Phe to 120·0, 42·0 and 35·9 mg/l, using respectively flavourzyme, alcalase and iZyme. However, using the double amount of enzyme (2 V) and 1 h of hydrolysis, the Phe concentrations achieved were only 125·8, 29·7 and 17·5 mg/l using respectively flavourzyme, alcalase and iZyme.

Fermentation of crude or enzymatically hydrolysed cheese whey

Crude cheese whey and hydrolysates obtained in previous enzymatic experiments were inoculated with strains of *L. plantarum* and tested for the production of PLA. The growth in absence of a metabolically relevant number of contaminations was double checked by examination of morphology in microscope and visual observation of uniform colonies on agar plates.

Cheese whey without enzymatic hydrolysis (crude cheese whey) was assayed as fermentation broth in absence or presence of the nutrients optimised by Brinques et al. (2010) for *L. plantarum*. During the fermentation of crude cheese whey, in absence of nutrients (F1), Phe was continuously released from the initial 4·3 ± 0·283 mg/l up to 104·3 ± 6·505 mg/l after 78 h (see Table 1). However, PLA was scarcely produced (0·017 ± 0·006 mM) to achieve a volumetric productivity (Q_{PLA}) of only 0·036 ± 0·012 mg/l·h, indicating that the

rate of Phe formation was not enough to fulfil the microorganism requirements for the formation of product. Vermeulen et al. (2006) reported that *Lactobacillus sanfranciscensis* hydrolysed 76–100% of the peptides supplied as dipeptides-containing phenylalanine, thus causing an accumulation of Phe in the course of fermentation. Similarly, *L. plantarum* hydrolysed dipeptides as well, increasing the Phe levels from 0·44 up to 1·38 mM after 72 h. Additionally, these authors also reported that peptide hydrolysis and PLA formation continued when growth had ceased, showing that amino acid conversion is not related to exponential growth but an ongoing process in stationary cells. Using this fermentation broth, lactose was not consumed, and consequently negligible amounts of organic acids were quantified.

The fermentation broth prepared with cheese whey and nutrients (F2) revealed that a considerable proportion of Phe comes from these nutrients, increasing the initial amount of Phe up to 152·9 ± 6·364 mg/l. This supplementary amount of Phe, stimulated the formation of PLA up to 0·93 ± 0·085 mM, and consequently Q_{PLA} to 2·188 ± 0·199 mg/l·h. Besides, from the high value of product yield calculated ($Y_{PLA/Phe}$ = 1·18 ± 0·102 g/g) it can also be concluded that Phe was also released during the process, as it happened in the previous fermentation. In this case, lactose was completely metabolised after 71 h, reaching a lactic acid concentration of 33·5 ± 0·566 g/l (Q_{LA} = 0·467 ± 0·031 g/l·h) and 4·3 ± 0·042 g/l acetic acid.

Considering the complexity of the culture medium, cheese whey hydrolysed during 1 h was assayed in the absence (F3, F4 and F5) or presence (F6, F7 and F8) of nutrients. As it can be seen in Table 1 the tendency was similar to that observed using crude hydrolysates, with increased PLA concentrations after supplementation with nutrients. iZyme, followed by alcalase and flavourzyme showed the highest concentration of PLA, with values of 1·02 ± 0·099, 0·77 ± 0·085 and 0·64 ± 0·113, respectively, in experiments performed after supplementation. With the only exception of hydrolysates obtained with flavourzyme (F4), where lactose was partially consumed to produce 17·0 ± 1·414 g/l lactic acid and 2·9 ± 0·157 g/l acetic acid, the absence of nutrients was unfavourable for fermentation. Conversely, after nutrient addition (F6–F8), lactose was completely consumed, yielding high levels of lactic (39·3 ± 0·990 to 41·7 ± 1·980 g/l) and acetic (4 ± 0·141 to 5 ± 0·283 g/l) acids.

The influence of enzymatic hydrolysis can be inferred comparing F2 (crude cheese whey supplemented with nutrients) with F6, F7 and F8 (cheese whey after enzymatic hydrolysis and supplementation with nutrients). Only a slight increment in PLA concentration was observed in F8 after enzymatic hydrolysis with iZyme, from 0·93 ± 0·085 up to 1·02 ± 0·099 mM. However, in all cases, the time required to achieve the maximal amount of PLA was considerably reduced, from 71 h to only 46–54 h. Therefore, it can be concluded a beneficial effect of enzymatic hydrolysis for the metabolic pathway.

Finally, two strategies were evaluated in order to improve the production of PLA: doubling the amount of enzyme required to hydrolyse 10 g/l of protein (F9, F10 and F11) or increasing the time of hydrolysis to 12 h (F12, F13 and F14).

The first strategy was detrimental resulting in a slight reduction in the amount of PLA (see Table 1), which could be due to an excess of enzyme was harmful, or that the large number of compounds released could inhibit the fermentation. In fact, using the enzyme Flavourzyme (F10), despite the higher value of initial Phe (278·7 ± 20·930 mg/l), the value of Phe at the end of fermentation increased even further to 287·8 ± 9·899 mg/l. Regarding the generation of lactic and acetic acids, no influence was observed using Alcalase (F9), since lactose was entirely depleted, bearing reasonable amounts of lactic acid (38·8 ± 0·566 g/l). However, using Flavourzyme or iZyme, lactose was not consumed at the end of fermentation and consequently no lactic acid was produced.

Conversely, the second strategy was more positive, and therefore the highest concentration of PLA (1·30 ± 0·071 mM) and global volumetric productivity (Q_{PLA} = 4·001 ± 0·218 mg/l·h) were attained in experiment F13 using the necessary amount of enzyme Flavourzyme to hydrolyse 10 g/l of protein, during 12 h. Similar results (PLA = 1·26 ± 0·113 mM; Q_{PLA} = 3·878 ± 0·348 mg/l·h) were achieved using the enzyme iZyme and 12 h of hydrolysis, in spite of starting with a lower amount of Phe (188·8 ± 13·435 mg/l) compared with the 272·9 ± 28·991 mg/l of Phe when using Flavourzyme. The calculated values of product yield also suggested a continuous release of Phe in the course of the fermentation. The results of PLA achieved after enzymatic hydrolysis during 1 h (F6, F7 and F8) or 12 h (F12, F13 and F14) confirmed that the metabolic pathway not only depends on the concentration of the biosynthetic precursor (phenylalanine), since these values are similar independently of the time of hydrolysis, but also supported the hypothesis that during the enzymatic hydrolysis, some compounds that may promote or stimulate the metabolic pathway of Phe into PLA, were also released. In these fermentations, lactose was effectively converted into lactic and acetic acids, according to the data summarised in Table 1.

Characterisation of raw and hydrolysed cheese whey

Raw cheese whey and cheese whey hydrolysed under optimised conditions (Flavourzyme at 50 °C during 12 h and 100 rpm) were characterised and the main parameters summarised in Table 2. The hydrolysis of cheese whey promotes the fractionation of proteins into smaller units. These compounds have more water biding sites, thus increasing the whey solubility (Sinha et al. 2007; Corrêa et al. 2014). This occurrence could explain the decrease in cheese whey total solids in our study from 63·5 ± 0·283 to 54·4 ± 0·460 g/l and proteins from 11·2 ± 0·011 to 8·2 ± 0·022 g/l. The increment of nutrients can be inferred from the higher quantification of amino acids and metals determined after cheese whey hydrolysis (see Table 2). All the amino acids content increased during the hydrolytic process with the only exception of histidine, arginine and proline. Due to the objective of this study, it

Table 2. Characterisation of crude cheese whey and cheese whey after enzymatic hydrolysis (Flavourzyme at 50 °C and 100 rpm during 12 h)

	Before	After	Increment (%)
pH	6·3 ± 0·013	7·0 ± 0·019	
Total solids (g/l)	63·5 ± 0·283	54·4 ± 0·460	−14·3
Lactose (g/l)	48·5 ± 0·207	49·5 ± 0·196	2·1
Lactate (g/l)	0·15 ± 0·165	0·21 ± 0·203	40·0
Protein (g/l)	11·2 ± 0·011	8·2 ± 0·022	−26·8
Fat (g/l)	3·7 ± 0·017	3·7 ± 0·028	0·0
Amino acids (g/l)			
Hydroxyproline	0·01 ± 0·00	0·01 ± 0·00	10·0
Aspartic acid	0·11 ± 0·02	0·16 ± 0·03	43·5
Serine	0·29 ± 0·08	0·36 ± 0·09	25·2
Glutamic acid	0·29 ± 0·07	0·45 ± 0·10	57·0
Glycine	0·05 ± 0·01	0·07 ± 0·02	31·4
Histidine	0·33 ± 0·08	0·19 ± 0·03	−43·4
Taurine	0·03 ± 0·00	0·04 ± 0·01	28·8
Arginine	0·15 ± 0·03	0·15 ± 0·04	−2·6
Threonine	0·18 ± 0·08	0·20 ± 0·09	11·4
Alanine	0·21 ± 0·09	0·27 ± 0·11	24·9
Proline	0·05 ± 0·01	0·05 ± 0·01	−1·2
Tyrosine	0·14 ± 0·04	0·16 ± 0·05	15·0
Valine	0·28 ± 0·08	0·33 ± 0·09	17·4
Methionine	0·16 ± 0·05	0·17 ± 0·04	7·6
Lysine	0·59 ± 0·11	0·74 ± 0·14	25·4
Isoleucine	0·31 ± 0·07	0·35 ± 0·05	11·6
Leucine	0·54 ± 0·14	0·65 ± 0·17	19·2
Phenylalanine	0·15 ± 0·04	0·18 ± 0·08	21·2
Metals (mg/kg)			
K	1·6 ± 0·04	2·0 ± 0·09	25·0
Cu	<1·8	<1·8	–
Zn	<1·0	<1·0	–
Fe	<6·0	<6·0	–
Mn	<1·2	<1·2	–
Ca	292 ± 23	362 ± 34	24·0
Mg	79·2 ± 8	77·5 ± 14	−2·1
Na	456 ± 31	915 ± 56	100·7
Al	<30	<30	–

is particularly noticeable the increment of 21·2% observed with phenylalanine. Regarding the study of metals, after hydrolysis, it was observed a strong increment in the amount of Na, K and Ca. Mg decreased slightly; meanwhile the remaining metals were hardly quantified. Rossini et al. (2009) demonstrated in their work the efficiently of Flavourzyme to hydrolyse the casein molecule. The major concentration of Ca, present in raw cheese, is partially bonded with casein micelles. When this structure is hydrolysed, normally the Ca concentration increased (Gaucheron, 2005).

Fermentation of cheese whey hydrolysates supplemented with vinasses

The solid or the freeze-dried liquid fractions of wine vinasses were evaluated as the only nitrogen source in order to increase the economical potential for larger-scale bioproduction. Salgado et al. (2009) suggested that, in spite of being one of the most common nitrogen sources in a variety of bioprocesses, the high cost of yeast extract impairs the economics, estimating that its value accounts for 38% of the final cost of lactic acid.

Figure 1 depicts the kinetics of Phe and lactose consumption as well as the generation of phenyllactic and lactic acids; meanwhile Table 3 summarises the parameters of fermentation. Using vinasses as a source of nitrogen, the concentration of PLA ranged between 1·15 ± 0·071 and 1·20 ± 0·052 mM, slightly lower to the value of 1·55 ± 0·060 mM achieved with commercial nutrients (used as control). Nevertheless, these values are comparable or superior to those reported by Vermeulen et al. (2006) using *L. sanfranciscensis* or *L. plantarum* (0·25–0·75 mM respectively); Li et al. (2007) using *Lactobacillus* sp. SK007 (0·55 mM); Rodríguez et al. (2012) using *L. acidophilus, Lactobacillus pentosus, L. plantarum, L. rhamnosus*, and *Lactococcus lactis* (0·17–1·38 mM) with the highest value working with *L. plantarum*; Valerio et al. (2004) during the screening of 28 strains (0·02–0·57 mM) or the 13 LAB screened by Cortés-Zavaleta et al. (2014) in culture tubes prepared with fresh sterile MRS broth (0·021–0·275 mM), although in this case, no PLA was quantified with *L. plantarum*.

The product yields oscillated between 0·53 ± 0·014 and 0·68 ± 0·460 g/g in all cases. The most outstanding result could be the fact that using 50 ml freeze-dried vinasses and 20 g/l solid vinasses, the time of fermentation was reduced to only 24 h, thus increasing the Q_{PLA} up to 0·008 ± 0·005 g/l·h and the volumetric rate of Phe consumption (Q_{Phe}) up to 0·0138 ± 0·0003 g/l·h. In contrast, lactose was efficiently converted in lactic acid in all fermentations, with product yields ranging from 0·70 ± 0·020 to 0·93 ± 0·059 g/g, although the higher lactic acid value (40·3 ± 0·778 g/l) was achieved in fermentations carried out using 100 ml freeze-dried vinasses as nutrients (fermentation d). Consequently, vinasses could be employed to overcome one of the major technical hurdles for the development of low-cost culture media that could stimulate the production of natural products. Taking into account not only the higher global volumetric productivities achieved for both lactic acid and PLA when using freeze-dried and solid vinasses, but also considering the higher profit of using both fractions, further experiments were conducted using this combination of vinasses as nutrients in continuous processes.

Continuous fermentation of cheese whey hydrolysates supplemented with vinasses

Finally, the process was scaled up to a 2 L Braun Biostat fermenter operating on continuous. Table 3 shows the results obtained under three steady states reached at selected dilution rates. As it was expected, under the lowest dilution rate assayed (0·0057 h^{-1}), although the maximal amount of PLA concentration was achieved (0·91 ± 0·085 mM), the process rendered a low Q_{PLA} (0·0086 ± 0·0001 g/l·h) and Q_{Phe} (0·0039 ± 0·0002 g/l·h) at a reasonable $Y_{PLA/Phe}$ (0·48 ± 0·007 g/g). The PLA concentration decreased continuously

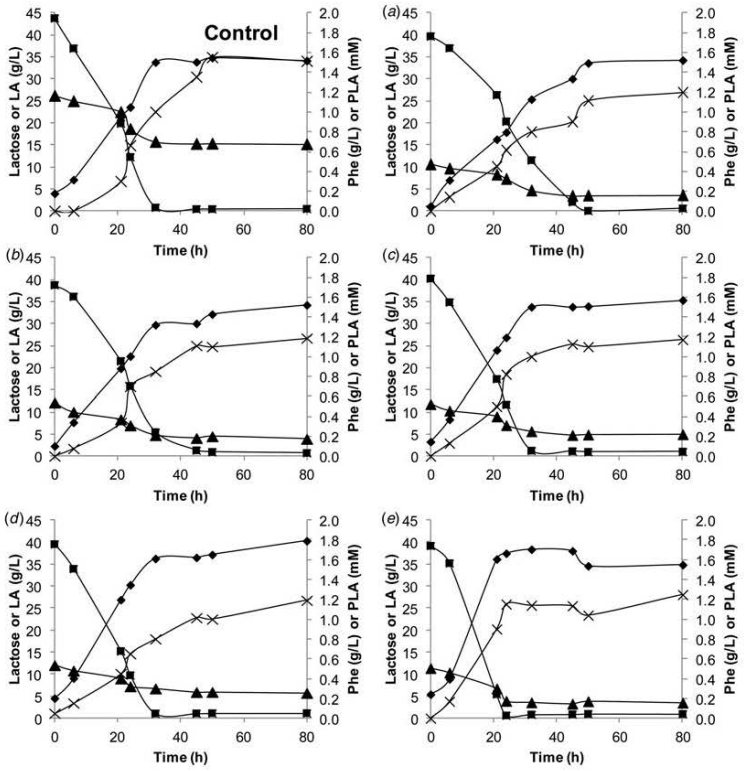

Fig. 1. Kinetics of Phe and lactose consumption, and PLA and lactic acid production using cheese whey hydrolysates as carbon source and vinasses as nitrogen source in fermentations carried out in Erlenmeyer flasks. (a) 25 ml freeze-dried vinasses; (*b*) 50 ml freeze-dried vinasses; (*c*) 75 ml freeze-dried vinasses; (*d*) 100 ml freeze-dried vinasses; (*e*) 50 ml freeze-dried vinasses and 20 g/l solid vinasses. Lactose (■); lactic acid (♦); Phe (▲); PLA (×).

when the dilution rate was increased; with the lowest PLA concentration (0·65 ± 0·127 mM) and $Y_{PLA/Phe}$ (0·17 ± 0·036 g/g) achieved under the highest dilution rate assayed (0·0393 h^{-1}). Under this dilution rate, the higher Q_{PLA} (0·0042 ± 0·0008 g/l·h) and Q_{Phe} (0·025 ± 0·0006 g/l·h) were achieved, meaning that it cannot be inferred an optimal value to work with.

Conversely, regarding the conversion of lactose into lactic acid, lactose was completely consumed in all cases (see Table 3); yielding similar lactic acid concentrations (38·4 ± 3·932 to 39·7 ± 1·980 g/l) corresponding to products yields of 0·85 ± 0·161 to 0·88 ± 0·012 g/g. However, an optimum value of Q_{LA} (1·563 ± 0·078 g/l·h) and $Q_{Lactose}$ (0·859 ± 0·460 g/l·h) was achieved under the highest dilution rate, considering that a similar lactic acid concentration was attained in a shorter period of time.

Antimicrobial effect of CFS

Finally, the antimicrobial activity of cell-free supernatants (CFS) was evaluated against *C. piscicola* as indicator microorganism. CFS extracts produced halos with mean values of 12·03 ± 0·50 mm. Commercial lactic acid, PLA and nisin (in the amounts present in CFS) were also evaluated as a control and the mean inhibitory halos depicted in Fig. 2. Using 41 g/l of commercial lactic acid, the inhibitory halo was 6·60 ± 0·63 mm, meaning that the effect of this commercial acid was equivalent to 54·9% of the value obtained with CFS. Conversely, the use of 0·81 mM of commercial PLA had no inhibitory effect. It was necessary to increase the amount of PLA up to 7·5 mM to observe a minimum effect of 0·10 ± 0·05 mm. However, commercial nisin with equivalent concentration (1·25 g/l) increased the inhibitory

Table 3. Summary of values obtained in the fermentation of cheese whey hydrolysates and vinasses under different fermentation conditions: using 250 ml Erlenmeyer flasks or 2 litres Bioreactor operating in continuous under three dilution rates (in h^{-1})

	Erlenmeyer flasks						Bioreactor		
	Control	(a)	(b)	(c)	(d)	(e)	0·0057 h^{-1}	0·0207 h^{-1}	0·0393 h^{-1}
Time (h)	50	80	80	80	80	24	–	–	–
$Phe_{t=0}$ (g/L)	1·2 ± 0·090^{b}	0·47 ± 0·071^{a}	0·54 ± 0·042^{a}	0·52 ± 0·113^{a}	0·53 ± 0·071^{a}	0·51 ± 0·049^{a}	–	–	–
Phe_{final} (g/l)	0·68 ± 0·040^{d}	0·16 ± 0·031^{a}	0·17 ± 0·027^{a}	0·22 ± 0·042^{ab}	0·26 ± 0·035^{abc}	0·18 ± 0·042^{a}	0·32 ± 0·042^{bc}	0·37 ± 0·014^{c}	0·36 ± 0·014^{bc}
$PLA_{t=0}$ (mM)	0	0	0	0	0	0	–	–	–
PLA_{final} (mM)	1·55 ± 0·060c	1·20 ± 0·052a	1·19 ± 0·082a	1·17 ± 0·085a	1·19 ± 0·092a	1·15 ± 0·071a	0·91 ± 0·085ab	0·81 ± 0·026b	0·65 ± 0·127b
Q_{PLA} (g/l·h)	0·0052 ± 0·0000^{b}	0·0025 ± 0·0001^{a}	0·0025 ± 0·0002^{a}	0·0024 ± 0·0002^{a}	0·0025 ± 0·0002^{a}	0·0080 ± 0·0005^{d}	0·00086 ± 0·0001^{c}	0·00028 ± 0·0001^{a}	0·0042 ± 0·0008^{b}
Q_{Phe} (g/l·h)	0·0104 ± 0·0010^{c}	0·0039 ± 0·0005^{a}	0·0046 ± 0·0002^{a}	0·0038 ± 0·0009^{a}	0·0034 ± 0·0004^{a}	0·0138 ± 0·0003^{b}	0·0039 ± 0·0002^{a}	0·013 ± 0·0003^{b}	0·025 ± 0·0006^{d}
$Y_{PLA/Phe}$ (g/g)	0·53 ± 0·029^{a}	0·64 ± 0·055^{ab}	0·53 ± 0·014^{a}	0·66 ± 0·109^{ab}	0·74 ± 0·040^{b}	0·58 ± 0·023^{ab}	0·22 ± 0·007^{c}	0·21 ± 0·002^{c}	0·17 ± 0·036^{c}
$Lactose_{t=0}$ (g/l)	43·7 ± 2·107^{a}	39·6 ± 2·263^{a}	38·7 ± 2·404^{a}	40·2 ± 0·085^{a}	39·5 ± 2·828^{a}	39·1 ± 1·909^{a}	45·1 ± 3·507^{a}	45·1 ± 1·612^{a}	45·1 ± 1·612^{a}
$Lactose_{final}$ (g/l)	0	0·55 ± 0·403^{a}	0·7 ± 0·240^{a}	1·01 ± 1·089^{a}	1·15 ± 0·523^{a}	0·63 ± 0·297^{a}	0	0	0
$LA_{t=0}$ (g/l)	4·0 ± 0·813^{ab}	1·1 ± 0·806^{c}	2·3 ± 0·085^{ac}	3·3 ± 0·849^{abc}	4·6 ± 0·099^{ab}	5·4 ± 0·778^{b}	–	–	–
LA_{f} (g/l)	34·8 ± 1·414^{a}	34·2 ± 1·131^{a}	34·2 ± 1·838^{a}	35·3 ± 1·838^{a}	40·3 ± 0·778^{a}	37·5 ± 1·838^{a}	38·4 ± 3·932^{a}	38·8 ± 3·253^{a}	39·7 ± 1·980^{a}
Q_{LA} (g/l·h)	0·616 ± 0·012^{c}	0·414 ± 0·024^{a}	0·399 ± 0·022^{a}	0·400 ± 0·012^{a}	0·446 ± 0·011^{a}	1·338 ± 0·044^{e}	0·219 ± 0·022^{b}	0·803 ± 0·067^{d}	1·563 ± 0·078^{f}
$Q_{Lactose}$ (g/l·h)	0·875 ± 0·042^{b}	0·491 ± 0·023^{a}	0·475 ± 0·027^{a}	0·490 ± 0·013^{a}	0·479 ± 0·042^{a}	1·603 ± 0·067^{d}	0·257 ± 0·020^{c}	0·932 ± 0·033^{b}	1·774 ± 0·063^{e}
$Y_{LA/Lactose}$ (g/g)	0·70 ± 0·020^{a}	0·84 ± 0·009^{a}	0·84 ± 0·002^{a}	0·82 ± 0·046^{a}	0·93 ± 0·059^{a}	0·83 ± 0·007^{a}	0·85 ± 0·161^{a}	0·86 ± 0·041^{a}	0·88 ± 0·012^{a}

Phe: phenylalanine; PLA: DL-3-Phenyllactic acid; Q_{PLA}: global volumetric productivity of PLA; Q_{Phe}: volumetric rate of Phe consumption; $Y_{PLA/Phe}$: Phe to PLA yield, calculated as $(PLA_{final}-PLA_{t=0})/(Phe_{t=0}-Phe_{final})$ without considering the amount of phenylalanine released in the course of fermentation; LA: lactic acid; Q_{LA}: global volumetric productivity of LA; $Q_{Lactose}$: volumetric rate of lactose consumption; $Y_{LA/Lactose}$: Lactose to LA yield. (a) 25 ml freeze-dried vinasses; (b) 50 ml freeze-dried vinasses; (c) 75 ml freeze-dried vinasses; (d) 100 ml freeze-dried vinasses; (e) 50 ml freeze-dried vinasses and 20 g/l solid.
Different letters mean statistically significant differences among values in the same line ($P < 0{\cdot}05$).

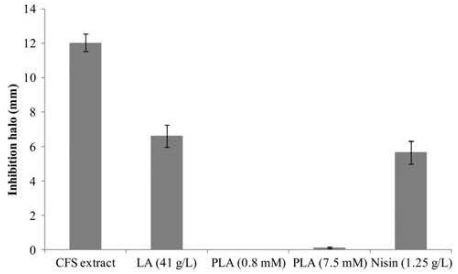

Fig. 2. Halos, expressed as a mean of three replications, induced by the cell-free supernatant of *Lactobacillus plantarum* CECT-221, commercial lactic acid (41 g/l), PLA (0·8 or 7·5 mM) or nisin (1·25 g/l), against the indicator microorganism *Carnobacterium maltaromaticum*.

halo up to 5·65 ± 0·65 mm. These results pointed out the contribution of all the compounds present in the CFS.

Although Lavermicocca et al. (2003) and Prema et al. (2008) found that PLA produced by *L. plantarum* showed inhibitory action against different species of moulds, other authors reported that the high minimum inhibitory concentration for commercial PLA is in the range of 3·01–36·10 mM against some food spoilage moulds. These values suggest that PLA cannot be considered the only compound related with the antifungal potential and that synergistic effects may exist among PLA and other products obtained during the metabolism of LAB (Cortés-Zavaleta et al. 2014). In this way, Tirloni et al. (2014) proposed that the antagonistic ability of LAB as biopreservants can be explained by the competition for nutrients and through the production of antimicrobial compounds such as bacteriocins, reuterin, organic acids (mainly acetic and lactic acids), carbon dioxide, diacetyl, ethanol, hydrogen peroxide and enzymes. In this context, Schwenninger et al. (2008) in addition to PLA also identified by chromatography and mass spectrometry the presence of propionic, acetic, and lactic acids, 2-pyrrolidone 5-carboxylic acid, hydroxyphenyllactic acid, and succinic acid during the co-culture of *Lactobacillus paracasei* and *Propionibacterium jensenii* in supplemented whey permeate medium.

In conclusion, cheese whey can be efficiently enzymatically hydrolysed to improve the availability of phenylalanine. Hydrolysates supplemented with vinasses can be fermented by *Lactobacillus plantarum* CECT-221 in batch or continuous fermentations using 2 litres Bioreactors. Cell-free supernatants showed antimicrobial activity although the results suggested that PLA was not the main metabolite responsible, being more important the contribution of lactic acid and bacteriocins present in the CFS.

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Review

Biosurfactant-enhanced hydrocarbon bioremediation: An overview

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ABSTRACT

The water and soil contamination by aromatic hydrocarbons has been increasing over the years, due to its use in several industrial segments. Hydrocarbons are described as extremely pollutant, toxic, with carcinogenic and mutagenic potential for humans. The concern with these compounds increases due to the difficulties in removing them from the environment. The remediation methods for contaminated environments are based on chemical, physical or biological activity. With the advance of sustainable technologies, the search for natural methods for the removal and/or degradation of soil and water contaminants has increased. Biosurfactants are surfactants produced mainly by microorganisms that promote the cracking of hydrocarbons molecules by micelle formation, increasing their mobility, bioavailability and exposure to bacteria, thus favoring hydrocarbon biodegradation. There is a great diversity of microorganisms that are capable of biodegrading pollutants such as oil and producing biosurfactants, but they are not well known. This study aims to address the issues related to a series of parameters involved in the production and in the mobilization and action mechanism of biosurfactant monomers in sites containing hydrocarbons.

Contents

1. Hydrocarbon contamination

Concerns related to the potential for soil and water contamination by oil and its byproducts are increasing, as they are one of the main contaminants in the environment. The contamination sources are diverse: accidents in fuel transportation by ships and trucks; leakages from underground storage tanks, which are subject to corrosion, as in gas stations; oil extraction and processing operations; and inadequate release of oily waste generated by industries that use oil byproducts in the production of plastics, solvents, pharmaceuticals and cosmetics (EPA – Environmental Protection Agency of United States, 2008; Lin et al., 2010).

Although petrochemical plants and oil refineries are beneficial to society, they produce a great quantity of solid oily waste (about 10,000 m^3 per day) classified as hazardous waste, which cannot be reused or recycled, as they are flammable, corrosive, toxic or pathogenic (Gafarov et al., 2006).

In refineries, oil is converted into autogas and diesel fuel. The gasoline consists of relatively volatile hydrophobic hydrocarbons such as alkanes, cycloalkanes, BTEX (benzene, toluene, ethylbenzene, and xylene), phenol and polycyclic aromatic hydrocarbons. Many of these compounds are described as highly pollutant,

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as they present a carcinogenic and mutagenic potential for humans, in addition to being toxic (Janbandhu and Fulekar, 2011). BTEX hydrocarbons have become a major concern in water pollution due to their toxicity and easy movement in the environment (Mazzeo et al., 2011).

According to the Environmental Sanitation Technology Company (CETESB, 1996) – the environmental control agency of the State of São Paulo, Brazil – leakages in gas stations accounted for 78% of the cases of contaminated areas in the State of São Paulo in 2011, which corresponds to 3217 areas.

About 90% of the gasoline hydrocarbons spilled into the environment, found in drinking water sources, are composed of BTEX, which raises concerns on environmental health because of their toxic, mutagenic and carcinogenic properties (Janbandhu and Fulekar, 2011).

When there are leakages in fuel tanks, hydrocarbons are dispersed and penetrate the soil. According to Bachu (2008), the migration of these compounds is regulated by the formation of four distinct phases, known as: Residual liquid phase – liquid waste are relatively immobile, adsorbed or retained in soil solids; Free liquid phase – without the presence of waste, they easily migrate through the soil and may reach groundwater; Dissolved phase – in this phase, the hydrocarbons form a layer on solid surface or soil water, and form a contamination plume when they reach groundwater; and Vapor phase – they join the soil vapor and may condense and adsorb on solid surface or dissolve in soil water.

The high soil–water mobility of these hydrocarbons is related to their low octanol–water partition coefficient, which ensures a slow absorption by the soil and allows its transportation by water, quickly moving through the soil, thus favoring water table contamination (Farhadian et al., 2008).

1.1. Risks posed by hydrocarbons

Soil and groundwater contamination by hydrocarbons present in fuels has become a focus of great concern both in industrialized and developing countries, due to its broad environmental distribution, which can reach soil, groundwater and air (Lebrero et al., 2012). BTEX hydrocarbons, present in the composition of gasoline and diesel fuel, are the first to reach the water table, as their constituents are highly soluble in water (Mariano et al., 2007). Consequently, these highly toxic compounds (except ethylbenzene) are the main causes of death by toxicity (Janbandhu and Fulekar, 2011).

Fellenberg (1980) reported that, when in contact with water, oil and its byproducts spread and form a thin layer on the surface that prevents gas exchange between air and water and blocks sunlight to phytoplankton, breaking the food chain. Confirming this theory, Asimiea and Sam-Wobo (2011) observed the impact of hydrocarbon waste on phytoplankton communities, which suffered structural changes due to the presence of these compounds.

One liter of oil can deplete the oxygen in one million liters of water and form a thin layer of 1000 m^2 on the soil surface in a few days, blocking the passage of sunlight and water and thus preventing the respiration and photosynthesis of the plants present therein (Yeung et al., 2011). Plants are prevented from performing respiration and photosynthesis due to a sealing the entrance of the stomata and plant roots are inhibited from absorbing soil nutrients (Fellenberg, 1980).

The marine environment has suffered with constant oil spills, making oil one of the most abundant organic contaminants in the sea. The media has been constantly denouncing the leakage of thousands of tons of oil that contaminate seawater (OESP, 2000–2013).

Half of world's oil production (around three billion tones/year) is transported by ships through the oceans, increasing hydrocarbon contamination levels in various marine ecosystems due to possible accidents. However, the major hydrocarbon source in the marine environment comes from routine operations of ship washing, natural oil leakages on sea bed and especially accidents in oil exploration and transportation (Marques Jr. et al., 2009).

One of the most impacting spills occurred recently, in November 2011, in the Sedco 706 oil platform, operated by Chevron Brasil in Campos Bay (Rio de Janeiro, Brazil). The oil leakage was equivalent to 5943 L and reached about 163 km^2 (ANP, 2011).

The potential threat to human health posed by hydrocarbons is connected to their physical and chemical properties, which allow these compounds to be absorbed by the skin and quickly spread through the organism if ingested or inhaled (Costa et al., 2012).

Exposure to BTEX hydrocarbons for a long period of time at low concentrations presents a series of chronic effects. Among them, benzene is regarded as the most toxic, and may cause depression of pluripotent primitive blood cells, extending through any cell maturation stage; damage to bone marrow, such as necrosis, edema, hemorrhage and fibrosis, which also interfere with blood cell production; leukemia and liver cancer. The estimated value of the minimum lethal dose (LDL) for humans is 194 mg kg^{-1} (Melo et al., 2007).

In 2000, Machado described the embryotoxicity of hydrocarbons and reported that women who had been exposed to high benzene levels in their professions had menstrual abnormalities, decrease in ovary size and potential fertility reduction.

Toluene presents moderate systemic toxicity to humans. If ingested, toluene is completely absorbed by the gastrointestinal system and is rapidly distributed to the body, mainly on adipose tissues, metabolized and excreted in the urine. When inhaled, this hydrocarbon compromises the central nervous system and may cause excitement or depression, with euphoria in induction stage, and later disorientation, tremors, fatigue, hallucinations, convulsions and coma. It may cause electrolyte abnormalities, metabolic acidosis, arrhythmias, muscle weakness; and causes mucosa irritation, transitional anomalies on liver enzyme activities and kidney problems. Moreover, embryotoxic and fetotoxic effects have been observed, though there are no clear evidences of teratogenic or carcinogenic activity in humans or laboratory animals (World Health Organization, 2006; Asimiea and Sam-Wobo, 2011).

Ethylbenzene and xylene, which have low systemic toxicity, are usually stored on adipose tissues and are almost completely metabolized and excreted in the urine. Both are central nervous system depressants; the first being a sensorineural irritant, and the latter, a skin and mucosa irritant. Studies carried out with both hydrocarbons have shown negative evidences for teratogenicity, carcinogenicity, metagenesis or genotoxicity (World Health Organization, 2006).

According to the EPA (2012), the specific chemical concentration to reach the risk level of a contaminant is called Risk-based concentration (RBC). According to the agency, in groundwater, benzene RBC is 2×10^{-4} (mg kg^{-1}), toluene RBC is 5.9×10^{-1} (mg kg^{-1}), ethylbenzene RBC is 1.5×10^{-3} (mg kg^{-1}), and xylene RBC is 1.9×10^{-1} (mg kg^{-1}). In 2001, the organization determined that the maximum BTEX component level in drinking water is 0.005 mg L^{-1} benzene, 1.0 mg L^{-1} toluene, 0.7 mg L^{-1} ethylbenzene and 10 mg L^{-1} xylene.

In 2006, the World Health Organization (WHO) determined that the maximum BTEX compound concentrations allowed in drinking water were: 0.01 mg L^{-1} benzene, 0.7 mg L^{-1} toluene, 0.3 mg L^{-1} ethylbenzene and 0.5 mg L^{-1} xylene. In Brazil, the maximum concentration of these hydrocarbons, established by the Brazilian National Environment Council for effluents discharge in water is 1.2 mg L^{-1} benzene and toluene, 0.84 mg L^{-1} ethylbenzene and 1.6 mg L^{-1} xylene (CONAMA, 2011).

In light of the problems caused by hydrocarbons, both for the environment and humans, the search for methods that contribute to removing or reducing these compounds in the environment has been increasing.

2. Remediation of the BTEX compounds

The compounds from the BTEX group, present in gasoline and diesel, present a higher solubility in water than the other components of these fuels, which explains the fact that, when an underground tank leaks, they are the contaminants found in larger amounts on the water table, being led by groundwater (Mariano et al., 2007). As approached, these hydrocarbons are dangerous to human health, creating a need to study methods that contribute to removing or reducing such compounds in the environment.

The methods for contaminated environment remediation are based on chemical, physical or biological means. Table 1 summarizes the main remediation techniques, as well as the possibly removed compounds (Coutinho and Gomes, 2007).

Currently, the most used method is Pump and Treat, in which the contaminated soil or water is removed by extraction wells that pump it to the surface in order to be treated by filter systems, volatile compounds extraction in aeration tower, or activated carbon, so they can be later rearranged. However, power and materials consumption for water and gas treatment are high, in addition to the expenses involved in the pumping system operation and maintenance and in the chemical analyses performance (CETESB/GTZ, 2001).

Table 1
Underground water and soil remediation techniques.

Technology	Description	Contaminants
Soil vapor extraction (SVE)	It physically removes organic compounds from the unsaturated zone by applying a vacuum system	CHC, BTEX
Bioventing	It speeds the removal process of volatile organic compounds by venting the vadose zone. It stimulates in-situ bioremediation	CHC, BTEX
Air sparging (AIS)	It physically removes volatile and semi-volatile compounds by venting the soil in the saturated zone. It stimulates in-situ bioremediation	CHC, BTEX, PAH, MTBE
Bio sparging	It speeds the organic compounds biodegradation by stimulating the native microflora, through physical venting processes of the saturated zone soil	CHC, BTEX, PAH, MTBE
Pump and treat	Physical process of extracting contaminated waters from the saturated zone and ex-situ treatment	CHC, BTEX
Thermal technology	In-situ thermal processes that destroy contaminants or speed the phase transference of the underground contaminant	CHC, BTEX
Accelerated bioremediation	It artificially alters the natural biochemical conditions of underground soil and water to speed degradation caused by microorganisms	CHC, BTEX
Phytoremediation	Appropriate plants are used to promote the extraction/degradation of organic compounds and heavy metals from soil	BTEX, CHC, PAH,
Soil washing/reinjection/chemical processes	Soil washing by proper fluid promotes extirpating and biodegradation. Chemical compounds (e.g. surfactants) can be added to speed the contaminants phase transfer	CHC, BTEX

Note: *CHC*: Chlorinated hydrocarbon compounds; *PAH*: Polycyclic Aromatic Hydrocarbons; *BTEX*: Petrol Hydrocarbons (Benzene, toluene, ethylbenzene, xylenes); *MTBE*: Methyl tert-butyl ether.
Adapted from Coutinho and Gomes (2007).

Chemical remediation is done by adding chemical compounds to deteriorate contaminants, i.e., to turn them into substances that are less toxic to the environment. The chemical reactions used include oxidation, reduction, polymerization or precipitation; oxidation being the most used method. The used oxidants are: ozone, peroxides, permanganate and persulfates. Even though it is a safe method with quick action, oxidant compounds are corrosive and may cause explosions if used in improper conditions and, at the end of the process, harmful substances to the local biota may still remain (CETESB/GTZ, 2001).

Chemical remediation is also applied on the soil washing technique, which uses surfactants that reduce the oil/water interfacial tension, in a way that the oil stays in solution, thus promoting the contaminant removal. This technique is applied *in situ*, though it is not quite used, as the surfactant may become another contaminant on the environment; or in reactors (CETESB, 1996).

The removal of these hydrocarbons can also be carried out by physical and chemical methods, such as the adsorption process. Activated carbon, used in this process, allows the recovery of the adsorbent and adsorbed, though it is a technique that requires many expenses (Daifullah and Girgis, 2003). Montmorillonite (a type of clay) has also been quite used for this purpose, as it has a low cost, does not present toxicity to the environment and is highly adsorbent; however, this clay has a hydrophilic nature, which renders it not effective in removing non-polar organic pollutants, which is the case of BTEX hydrocarbons (Gil et al., 2011).

With the advance of sustainable technology, the search for natural methods for the removal and/or degradation of soil and water contaminants has increased. The removal of these pollutants can be reached by biological treatment, which, in comparison with physical and chemical treatments, can provide advantages. These advantages include low operation cost, low power consumption and the absence of residual products (Shim et al., 2009).

The phytoremediation process is carried out with plants that are capable of degrading, extracting, containing or immobilizing soil and water contaminants (CETESB/GTZ, 2001). There are several types of phytoremediation:

a. *Phytoextraction and phytovolatilization*: processes that use plants called hyperaccumulators, which absorb, remove and dislocate soil contaminants to plant roots and upper parts.
b. *Phytostabilization*: uses plants that immobilize soil contaminants by sorption and precipitation in the rhizosphere.
c. *Phytodegradation*: promotes the degradation of organic contaminants by stimulating the microbial activity in the rhizosphere.
d. *Rhizofiltration*: process in which the sorption or precipitation of contaminants that are in solution around the roots occur, due to biotic or abiotic processes.

Besides phytoremediation, there is another process based in the use of living organisms to remediate hydrocarbon-contaminated sites, called Bioremediation. This technique consists in the biological decomposition of hydrocarbons by microorganisms that use these pollutants as a carbon source to obtain energy, biodegradating them into carbon dioxide, water, mineral salts and gases (Banat et al., 2010).

The larger the degrading microorganism population is, the quicker and more efficient the bioremediation process will be. Therefore, this technique can be carried out by biostimulation, which consists in the growth stimulation of the microorganisms

present at the contaminated site. This process can be carried out by introducing oxygen, nutrients and electron receptors specific for the degradation of the contaminant and substances in order to correct the pH. It can also be made by bioaugmentation, in which indigenous microorganisms (allochthonous) are added to the contaminated environment to accelerate and complete the pollutant degradation (CETESB/GTZ, 2001).

Low solubility and high hydrophobicity of hydrocarbons make them highly unavailable to microorganisms. Microorganisms produce biosurfactants in order to degrade hydrocarbons and use them as carbon source (Desai and Banat, 1997).

Biosurfactants are surfactants synthesized as metabolic products of different microorganisms, mainly aerobic ones, such as bacteria, yeasts and filamentous fungi using several substrates such as carbohydrates, hydrocarbons, oils and fats, industrial and agricultural residues or a mix of them (Pirôllo et al., 2008).

A property of great importance to industrial and biotechnological applications is the stability of most biosurfactants in different conditions, enduring high autoclaving temperatures (121 °C for 20 min) and also low temperatures (−18 °C for 6 months), and pH (5–11) found in the environment (Muthusamy et al., 2008).

According to Pirôllo (2006), the main biosurfactant classes currently known are glycolipids (rhamnolipids, sophorolipids, trehalose lipids), lipopeptides and lipoproteins (peptide, viscosin, serrawettin, surfactin, subtilisin, gramicidin, polymyxin), fatty acids, neutral lipids and phospholipids (fatty acids, neutral lipids and phospholipids), polymeric surfactants (emulsan, biodispersan, liposan, carbohydrate-lipid-protein, mannan-lipid-protein) and particulate surfactants.

Biosurfactants present many advantages in comparison with synthetic surfactants, such as high biodegradability, low toxicity, biocompatibility, biodigestibility (which allows their application in cosmetic and pharmaceutical products and as food additives), possibility to be produced from low-cost sources and industrial waste, use in bioremediation of oil-affected sites, biodegradation and detoxification of industrial effluents, in addition to efficacy in extreme temperature, pH and salinity conditions (Pirôllo, 2006).

3. Biosurfactant

With the advance of sustainable technologies, the search for natural, biodegradable compounds to remediate hydrocarbon-contaminated sites has been increasing. This search has led to the discovery that many surfactants can be of natural origin, being part of several cellular structures and biological membranes. Most of these surfactants are synthesized by living organisms, such as: saponins, produced by plants; glycolipids, by microorganisms; and bile salts, from animals. These compounds with surfactant properties produced by microorganisms are called biosurfactants (Aparna et al., 2011).

Biosurfactants have countless advantages in comparison with chemical surfactants, especially regarding biodegradability, compatibility with the environment, low toxicity, high selectivity and their activity even in extreme temperature, pH and salinity conditions (Banat et al., 2010). These compounds are surfactants and have amphipathic molecules, with hydrophobic and hydrophilic portions that act between fluids of different polarities (oil/water and water/oil), allowing access to hydrophobic substrates and causing changes such as surface tension reduction, and increasing the area of contact of insoluble compounds (such as hydrocarbons), their mobility, bioavailability and, later, its biodegradation (Aparna et al., 2011).

Biosurfactants have lipophilic groups of proteins and/or peptides with hydrophobics parts or carbonated chains of 10–18 carbons, and hydrophilic groups characterized by esters, hydroxyl, phosphate, carboxyl or carbohydrates. They are usually produced in the exponential phase or stationary phase of microbial growth, when there is a high cellular density (Suwansukho et al., 2008).

The biodegradation by biosurfactants of oil-derived hydrocarbons occurs by two mechanisms. The first includes the increase of biological availability of the hydrophobic substrate to microorganisms, with consequent surface tension reduction of the medium around the bacterium and interfacial tension reduction between the bacterial cell wall and hydrocarbon molecules. The other mechanism involves the interaction between biosurfactant and cell surface, promoting modifications in the membrane, facilitating hydrocarbon adherence (hydrophobicity increase) and reducing the lipopolysaccharide index of the cell wall without damaging the membrane (Aparna et al., 2011).

Thus, biosurfactants block the formation of hydrogen bridges and allow hydrophobic–hydrophilic interactions, which cause molecular rearrangement and reduce the surface tension of the liquid, increasing its surface area and promoting bioavailability and consequent biodegradability (Aparna et al., 2011).

Consequently, the biosurfactant production is indirectly evaluated by surface tension, which is related to the forces of attraction and repulsion between the molecules of a fluid. In order to keep the molecules cohesive on the liquid surface, they exert a strong intermolecular force of attraction on the molecules that are closer to the surface, called surface tension. Hence, the liquids tend to decrease their surface area, creating spherical drops that present lower surface/volume ration (Pirôllo et al., 2008).

Physically speaking, the surface tension would be the necessary energy to dilate the surface of a liquid. The higher the cohesion between liquid molecules, the higher the work required to increase the distance between them will be, i.e., the higher the force of attraction between the molecules is, the higher the surface tension will be (Pirôllo et al., 2008).

3.1. Action mechanism and biosurfactants efficiency

When biosurfactants are released, their monomers organize spherically (micelles), in a way that the hydrophobic portion is turned to the center, composing the nucleus, and the hydrophilic part is turned to the sphere surface, making an interface with water. Thus, the surfactant reduces the surface tension between water and oil and contributes to micelle formation, increasing hydrocarbon exposure to bacteria and oxygen and favoring hydrocarbon biodegradation (Soberón-Chávez and Maier, 2010).

The modifications in the membrane, such as change in proteins composition or increase of the cell wall hydrophobicity by the reduction of lipopolysaccharides, caused by the biosurfactant, promote higher accessibility to hydrocarbons by microbial cells. This occurs due to the dispersion of the hydrocarbon through its encapsulation in micelles, spherical or irregular vesicles and lamellar structures (Aparna et al., 2012).

The representation of micelle formation follows the Stigter's scheme, on which surfactant monomers organize spherically with the hydrophobic part composing the nucleus, and the hydrophilic portion on the external part of the grouping, making an interface with water. In a micelle, on the hydrophilic end of the biosurfactant, which makes an interface with water, a double, compact, electric layer that surrounds the external surface of the micelle sphere is formed and is called Stern Layer (Tondo et al., 2010).

The biosurfactant efficiency is determined by critical micelle concentration (CMC), the point in which micelles start to form (Soberón-Chávez and Maier, 2010). These aggregates are produced as a result of several weak chemical interactions, such as Van der Waals and hydrogen bridges. Micelle formation leads to the

Table 2
Biosurfactants, producing organisms and their uses.

Microorganism	Type of biosurfactant	Uses
Rhodococcus erythropolis 3C-9	Glycolipid and Trehalose Lipid	Oil spill cleaning operations
Pseudomonas aeruginosa S2	Rhamnolipid	Bioremediation of places contaminated by petroleum
Pseudozyma siamesis CBS 9960	Mannosylerythritol lipid	Promising yeast biosurfactant
Pseudozyma graminicola CBS 10092	Mannosylerythritol lipid	Washing detergent
Pseudomonas libanesis M9-3	Lipopeptide	Environmental and biomedical uses
Bacillus subtilis ZW-3	Lipopeptide	Pharmaceutical, environment protection, cosmetics and petroleum recovery
Rhodococcus sp. TW53	Lipopeptide	Bioremediation in sea environment
Pseudozyma hubeiensis	Glycolipid	Bioremediation in sea environment
R. wratislaviensis BN 38	Glycolipid	Bioremediation uses
Bacillus subtilis BSS	Lipopeptide	Bioremediation of places contaminated by hydrocarbon
Azobacter chroococcum	Lipopeptide	Environmental uses
Pseudomonas aeruginosa BS20	Rhamnolipid	Bioremediation of places contaminated by hydrocarbon
Micrococcus luteus BN56	Trehalose tetraester	Bioremediation of places contaminated by petroleum
Bacillus subtilis HOB2	Lipopeptide	Petroleum recovery, bioremediation of soil and sea environments and food industry
P. aeruginosa UFPEDA 614	Rhamnolipid	Bioremediation
Nocardiopsis alba MSA10	Lipopeptide	Bioremediation
Pseudoxanthomonas sp. PNK-04	Rhamnolipid	Environmental uses
Pseudozyma parantarctica	Mannosylmannitol lipid	Detergent or washing emulsifiers
Pseudomonas alcaligenes	Rhamnolipid	Environmental uses
Pseudomonas koreensis	Lipopeptide	Biologic control agent
Pseudomonas fluorescens BDS	Lipopeptide	Bioremediation and Biomedicine
Candida bombicola	Sorolipideos	Environmental uses
Brevibacterium aureum MSA13	Lipopeptide	Petroleum recovery
*Nocardiopsis lucentencis*MSA04	Glycolipid	Bioremediation in sea environment
Bacillus velezensis H3	Lipopeptide	Industrial strain for lipopeptide production
Calyptogena soyoae	Mannosylerythritol lipid	Bioremediation in sea environment
Burkholderia plantari DSM 9509	Rhamnolipid	Pharmaceutical and detergent industries

Source: Makkar et al. (2011).

reduction in surface and interfacial tension, due to the decrease in the force of repulsion between immiscible liquid phases (Aparna et al., 2012).

The molecule mobilization mechanism, which provokes the reduction of surface tension between air/water and soil/water, occurs in biosurfactant concentrations below CMC. The solubilization process starts in concentrations above CMC due to the formation of micelles that increase oil solubility (Urum and Pekdemir, 2004). The emulsification process starts when there is biosurfactant accumulation that forms a solution containing tiny droplets of oil suspended in aqueous medium. The high molecular weight of biosurfactants makes them efficient emulsifying agents. They are also used as additives to stimulate hydrocarbon bioremediation and removal from the environment (Franzetti et al., 2010).

An efficient biosurfactant has low CMC, in other words, a lesser quantity of surfactant is necessary to decrease surface tension, showing a greater effectiveness and efficiency than in chemical surfactants (Desai and Banat, 1997; Aparna et al., 2012). Surfactin, one of the most efficient biosurfactants known, reduces water surface tension from 72 mN/m to 27 mN/m, which is close to the minimum detectable value (Seydlová and Svobodová, 2008).

Biosurfactant formation and accumulation is related to a series of parameters that, according to Mukherjee et al. (2006), need to be more studied and comprehended in order to have more efficiency when producing these compounds with lower costs. The promising future of biosurfactants depends on the use of low cost raw-material and the optimization of growth conditions in order to better obtain the product.

4. Biosurfactant producing microorganisms

The studies on the use of hydrocarbons by microorganism began around 1906, performed by Sohnger and Kaserer. After a few years, in 1913, Sohnger proved that some microorganisms, mainly from the genera *Mycobacterium* and *Pseudomonas*, were capable of oxidizing gasoline, kerosene, paraffin and paraffin oil, CO_2, water and traces of organic acids (Zobell, 1946).

Thereafter, several studies have been performed in search of species capable of degrading aromatic compounds. In 1928, microorganisms from the genera *Micrococcus*, *Mycobacterium*, *Bacterium*, *Bacillus* and *Spirillum*, capable of degrading naphthalene, toluene, cresol and phenol, were isolated by Gray and Thornton (1928).

In 40 years, over 100 species from 30 microbial genera existing in nature were described for their capacity to use hydrocarbons as their sole carbon and energy source (Zobell, 1946).

Since the 90's, several microorganism genera were identified as hydrocarbon degraders: *Acinetobacter* sp., *Bacillus* sp., *Escherichia coli*, *Micrococcus luteus*, *Nocardia* sp., *Pseudomonas* sp., *Rhodococcus* sp., *Streptomyces* sp., *Vibrio* sp. *Xanthomonas maltophilia*, among others (Mariano et al., 2007).

According to Weltler-Tonini et al. (2010), the main bacteria genera capable of degrading oil byproducts are: *Acidovorans*, *Acinetobacter*, *Agrobacterium*, *Alcaligenes*, *Aeromonas*, *Arthrobacter*, *Beijemickia*, *Burkholderia*, *Bacillus*, *Comomonas*, *Corynebacterium*, *Cycloclasticus*, *Flavobacterium*, *Gordonia*, *Microbacterium*, *Moraxella*, *Mycobacterium*, *Micrococcus*, *Neptunomonas*, *Nocardia*, *Paracoccus*, *Pasteurella*, *Polaromonas*, *Pseudomonas*, *Ralstonia*, *Rhodococcus*, *Sphingomonas* and *Stenotrophomonas*.

In addition to bacteria, actinomycetes and fungi are also biosurfactant producers. However, when the aromatic hydrocarbon biodegradation by fungi occurs, trans-diols – potent carcinogens – are formed, while the biodegradation by bacteria produces cis-diols, which are not biologically active and do not result in carcinogenic potential (Atlas, 1995).

Since then, nine bacteria groups have been found in oil samples from oil reservoirs, such as: *Acinetobacter*, *Arcobacter*, *Bacillus*, *Halanaerobium*, *Leuconostoc*, *Marinobacter*, *Streptomyces*, *Propionibacterium* and *Streptococcus* (Sette et al., 2007).

Specifically, in 1908, the ability of microorganisms to degrade oil-derived hydrocarbons from the BTEX group was revealed, when

the *Bacillus hexabovorum* bacterium, isolated by Stormer, was proven capable of growing aerobically in a medium containing toluene and xylene (Zobell, 1946). In 1928, Gray and Thornton found, among 245 bacteria species isolated from non-contaminated soils, 146 which had the capacity to degrade BTEX hydrocarbons.

Biosurfactants are metabolites with surface activity produced by microorganisms in aqueous solutions containing hydrophobic compounds (Mukherjee et al., 2006). These surfactants comprise different structures and chemical compositions that vary depending on the producing microorganism, the nutrients from the culture medium and the growth conditions. Mainly, they consist of fatty acids, glycolipids, lipopeptides, lipopolysaccharides and lipoproteins (Makkar et al., 2011) (Table 2).

Pseudomonas is known for its capacity to produce extensive quantities of glycolipids. These produced biosurfactants are classified as Rhamnolipids, capable of reducing water surface tension to values between 25 and 30 mN/m, with CMC (critical micelle concentration) between 10 and 230 mg/L, depending on the different microbial sources and the pH and salinity conditions of the medium (Aparna et al., 2011).

Another microorganism broadly studied on its biosurfactant production is the *Bacillus subtilis*, known for its confirmed efficiency in producing a lipopeptide with surface activity, named surfactin or subtilisin - one of the most potent biosurfactants known. It reduces water surface tension from 72 to 27 mN/m, with CMC in 24 mM water (Desai and Banat, 1997; Al-Bahry et al., 2013).

Due to the great results obtained with surfactin, the biosurfactant production by other species of *Bacillus* has been studied. Yakimov et al. (1995) published a work on the production of a new biosurfactant by *Bacillus licheniformis*, named lichenysin, which has demonstrated great stability in different temperatures, pH and salt concentrations. Additionally, it has shown the capacity to reduce water surface tension from 72 mN/m to 28 mM, with CMC less than or equal to 12 mM. Recent studies revealed that some strains of *Bacillus megatherium* also produce biosurfactants, classified as glycolipids (Thavasi et al., 2011) or lipopeptides, with the capacity to reduce water surface tension to 28–30 mN/m, with approximately 100 mg/L CMC (Pueyo et al., 2009).

The diversity of microorganisms capable of biodegrading pollutants, such as oil, and producing biosurfactants is vast and little known. Depending on the studied habitat, it is estimated that less than 0.1% and a maximum of 10% of the existing microbial species have been discovered and named. However, the number of identified species, with advances on research, grows every year (Van Hamme et al., 2003).

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Chemical Engineering & Technology Research Article 1

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Volumetric Oxygen Mass Transfer Coefficient and Surface Tension in Simulated Salt Bioremediation Media

Toluene can be removed from contaminated sites via bioremediation through the addition of biosurfactant compounds, which reduce the surface tension. However, aeration and mixing must be optimized to ensure an effective volumetric oxygen mass transfer coefficient (k_La). Experiments were perfomed with different salt containing solutions, which were tested either as such, or with different supplements. k_La values obtained at different agitation intensities and aeration rates were compared with those in water, and correlated with power number and superficial gas velocity. Surface tension decreased when surfactin was added to toluene-containing media. The seawater-simulating medium exhibited the highest surface tension reduction.

Keywords: Aeration, Agitation, Oxygen mass transfer coefficient, Surface tension, Toluene

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1 Introduction

Accelerated industrial growth and lack of appropriate sites for chemical waste disposal have increased soil and water contamination. Particularly, aromatic hydrocarbons such as toluene, which are produced in large amounts by the oil industry, are often inadequately managed and illegally dumped, contaminating soil, groundwater, surface water, seawater, biota, and even air [1, 2]. According to Yeung et al. [3], one liter of oil can deplete oxygen from one million liters of water and form a 1000 m^2 thin layer in a few days, thereby preventing light and water passage, and inhibiting plant and microbial growth.

Potentially harmful effects to human health caused by hydrocarbons are linked to their physicochemical properties such as lipophilicity, which enables them to be absorbed by the skin and distributed throughout the body when ingested or inhaled [4]. Besides provoking abnormal enzyme activity in the liver and kidney, toluene affects the human central nervous system, causing excitement or depression with euphoria at the induction phase, followed by disorientation, tremors, hallucinations, convulsions, and coma [5, 6].

Toluene is a monoaromatic hydrocarbon, ideal for biodegradation studies, which is produced in large quantities and used as a fuel, solvent, or raw material to produce plastics, synthetic fibers, and pesticides [1]. Usually occurring in mixtures, toluene and other monoaromatics have become prevalent environmental contaminants due to spills and leakage from tanks and other containers.

Oil-polluted sites can be decontaminated via so-called bioremediation. This technique, which can be performed either in situ or ex situ, consists of biodegradation of pollutants by microorganisms that utilize them as carbon and energy source, mainly releasing carbon dioxide and water [7]. However, the low solubility and high hydrophobicity of many hydrocarbons make them scarcely available to microorganisms, which are forced to release substances called biosurfactants to uptake and utilize them as carbon sources [8].

The volumetric oxygen mass transfer coefficient (k_La)[1)] is the most important parameter in all the aerobic bioprocesses [9], which is influenced by a number of factors, among which are the agitation intensity, aeration, bioreactor features, media composition, biomass concentration, and microbial species [10–14]. However, above all, it plays a key role in bioreactors, where it influences the level of oxygen available to aerobic metabolism; k_La may then be used as an indicator of performance of aerobic wastewater treatments as well as of bioremediation processes [15].

Biological processes are usually performed in culture broths, containing salts, organic nutrients such as sugars, hydrocarbons or alcohols, and sometimes surfactants, which are far more complex than clean water [16]. Therefore, studies on gas-liquid mass transfer in bioprocesses have become increasingly necessary [17]. Several authors have recently reported that advancing bioreactor designs is still of high priority to solve environmental problems and increase oxygen transfer from the gas phase to microorganisms [17, 18].

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1) List of symbols at the end of the paper.

Biosurfactants are wholly or partly extracellular polymers, produced by some microorganisms to make the uptake of lipophilic compounds easier, which accumulate at interfaces with different polarities. This feature prevents the formation of hydrogen bonds and certain hydrophobic-hydrophilic interactions, causing molecular rearrangements and reducing the surface tension, a property related to the attraction and repulsion forces among molecules of a fluid [8]. For this reason the release of biosurfactants such as surfactin is often indirectly assessed as a decrease in surface tension [19].

In this framework, in this study, the effects of aeration and agitation on k_La were investigated in media with different salt composition and osmolarity, either in the presence or the absence of toluene as a model pollutant, or of glucose or yeast extract as carbon and nitrogen sources, for their possible use in aerobic bioremediation processes. Moreover, the influence of different concentrations of surfactin on surface tension was investigated either with or without toluene. Understanding these effects is in fact an essential requisite to perform future work devoted to the selection of optimal conditions to maximize the activity of hydrocarbon-degrading microorganisms to be used in bioremediation processes.

2 Materials and Methods

2.1 Media Composition

Four media with different salinity to be used in future bioremediation processes were tested, namely, the basal saline medium (BSM), the low saline medium (LSM), the Bushnell-Haas medium (BHM), and a medium simulating seawater (SW), whose compositions are detailed in Tab. 1. The culture media was supplemented with glucose, yeast extract, and toluene as described in Tab. 2.

2.2 Bioreactor

All the experiments were carried out at 30 ± 1 °C in a 3.0-L bench-scale bioreactor, model BioFlo®/Celligen®115 (New Brunswick, Enfield, CT, USA), equipped with an electronic device to control temperature, dissolved oxygen level, rotational speed, and aeration rate. The main bench-scale design details were as follows: 1.0 L operating volume, 12 cm internal diameter, 18.5 cm height, 3 baffles, 14 cm baffle height, 2.3 cm baffle width, 2 impellers of the Rushton disc turbine type, 12 cm impeller distance from the top plate, 5 cm impeller distance from the bottom plate, 5.0 cm impeller disc diameter, 1.5 cm impeller blade width, 6 blades, 1.5 cm impeller blade length.

2.3 Osmolarity

Osmolarity of media, expressed in mOsmol L^{-1}, was measured using an automatic cryoscopic osmometer, model Osmomat 030 (Gonotec, Berlin, Germany), by determining the freezing point of 50-μL samples. The equipment was calibrated with a NaCl standard solution with osmolality of 300 Osm kg^{-1} using distilled water as a control.

2.4 k_La Determination

The volumetric oxygen mass transfer coefficient (k_La) was determined without any inoculum in all the above media, either as such or supplemented with 20 g L^{-1} glucose, 50 g L^{-1} yeast extract or 3 vol % toluene, under different aeration rates (0.5, 1.0, and 1.5 vvm) and agitation intensities (50, 100, 200, and 300 rpm).

To this purpose, dissolved oxygen was firstly removed by injecting nitrogen and the decrease in its concentration was mea-

Table 1. Composition of culture media: medium simulating seawater (SW), basal saline medium (BSM), Bushnell-Haas medium (BHM), and low saline medium (LSM).

Composition [mg/L]							
SW		BSM		BHM		LSM	
NaCl	27.6	$MgSO_4 \times 7H_2O$	50.0	$CaCl_2 \times 2H_2O$	23.0	$CaCl_2 \times 2H_2O$	2.3
$MgSO_4 \times 7H_2O$	6.9	$CaCl_2 \times 2H_2O$	1.0	$MgSO_4$	20.0	$MgSO_4$	2.0
$MgCl_2 \times 6H_2O$	5.4	KCl	10.0	KH_2PO_4	61.0	KH_2PO_4	6.1
$CaCl_2 \times 2H_2O$	1.4	$NaNO_3$	40.0	K_2HPO_4	55.0	K_2HPO_4	5.5
KCl	0.6	KH_2PO_4	50.0	NH_4NO_3	50.0	NH_4NO_3	5.0
$NaHCO_3$	0.2	K_2HPO_4	65.0	$FeCl_3$	69.0	$FeCl_3$	6.9
KBr	0.03	$FeSO_4 \times 7H_2O$	10.2	–	–	–	–
$SrCl_2 \times 6H_2O$	0.02	–	–	–	–	–	–
$MnSO_4 \times H_2O$	0.01	–	–	–	–	–	–
$NaHPO_4$	0.01	–	–	–	–	–	–
$Na_2MoO_4 \times 2H_2O$	0.001	–	–	–	–	–	–

Table 2. Values of volumetric oxygen mass transfer coefficient (k_La, s^{-1}), agitation intensity (rpm), aeration rate (vvm) and osmolarity ($mOsm\ L^{-1}$) in water (W), basal saline medium (BSM), low saline medium (LSM), Bushnell-Hass medium (BHM), and medium simulating seawater composition (SW). Samples supplemented with 20 g L^{-1} glucose (Glu), 50 g L^{-1} yeast extract (YE), 3 vol% toluene (Tol).

Medium	$k_La\ 10^3\ [s^{-1}]$ rpm / vvm	50	100	200	300	Osmolarity ($mOsmol\ L^{-1}$)
W	0.5	3.6	4.3	7.6	18.8	0
	1.0	5.3	6.2	10.0	14.6	
	1.5	7.1	9.6	11.7	18.8	
BSM	0.5	3.2	3.6	5.0	7.4	104
	1.0	5.5	5.1	7.8	13.3	
	1.5	7.6	7.8	10.1	16.5	
BSM + Glu	0.5	4.2	4.4	6.0	10.2	229
	1.0	6.3	6.0	8.7	14.9	
	1.5	8.1	8.3	11.0	17.7	
BSM + YE	0.5	2.6	2.7	3.9	6.8	452
	1.0	2.9	3.6	5.9	12.8	
	1.5	4.8	7.7	8.6	11.4	
LSM	0.5	4.1	2.9	3.4	8.6	11
	1.0	6.0	8.0	11.1	18.0	
	1.5	7.8	7.4	7.5	16.3	
LSM + Glu	0.5	4.5	3.0	6.3	9.8	130
	1.0	5.5	5.6	9.4	13.9	
	1.5	7.9	7.3	8.9	16.6	
LSM + YE	0.5	3.4	3.5	3.5	7.2	323
	1.0	1.6	4.7	6.2	9.1	
	1.5	4.6	5.3	7.6	11.0	
BHM	0.5	4.1	4.2	8.6	8.0	110
	1.0	5.8	5.9	11.1	16.0	
	1.5	9.9	10.4	13.2	19.7	
BHM + Glu	0.5	3.9	4.2	6.4	10.6	247
	1.0	6.9	6.8	9.2	16.1	
	1.5	8.7	9.1	10.7	18.3	
BHM + YE	0.5	3.1	3.1	4.1	9.4	359
	1.0	4.4	4.9	6.3	9.4	
	1.5	6.3	7.5	8.5	12.2	
SW	0.5	4.9	4.3	6.9	10.1	1100
	1.0	6.4	7.0	9.6	15.5	
	1.5	8.5	8.4	11.6	17.7	
SW + Glu	0.5	3.3	4.0	6.0	9.3	1275
	1.0	6.0	6.4	9.6	14.9	
	1.5	7.9	8.5	12.8	20.7	
SW + YE	0.5	3.1	3.7	5.5	9.3	1386
	1.0	4.4	4.9	6.4	12.9	
	1.5	5.1	6.0	7.3	14.2	
BSM + Tol	0.5	2.6	2.9	5.0	9.8	162
	1.0	4.9	5.1	6.5	13.0	
	1.5	6.6	6.7	8.3	14.7	
LSM + Tol	0.5	4.0	2.7	6.0	9.1	65
	1.0	4.9	3.0	10.6	16.1	
	1.5	4.8	7.2	11.5	16.8	
BHM + Tol	0.5	1.8	2.2	5.1	10.1	107
	1.0	5.5	5.1	5.4	15.2	
	1.5	5.3	6.9	6.9	17.0	
SW + Tol	0.5	2.4	1.2	4.1	7.6	1088
	1.0	2.8	3.7	6.1	9.8	
	1.5	4.6	6.7	12.1	15.9	

sured through an oxygen probe with a response time (τ) no longer than 10 s. There are several reports in the literature which support a satisfactory accuracy of dissolved oxygen determinations when $\tau << 1/k_La$ [20, 21] as in the present case. After the oxygen concentration reached approximately 0 % of saturation at 30 °C, N_2 injection was stopped and air bubbling was restarted. Oxygen concentration was newly monitored every 30 s until reaching approximately 100 % saturation (saturation point).

According to the literature [22], the mass balance of dissolved oxygen in well-mixed liquid systems can be described as:

$$\frac{dC}{dt} = k_La(C^* - C) \quad (1)$$

where dC/dt is the rate of O_2 accumulation in the liquid phase ($g\,m^{-3}\,s^{-1}$), C the oxygen concentration measured by the probe, and C^* the oxygen saturation concentration at equilibrium ($g\,m^{-3}$).

Integration of this equation between $t = 0$ and t, and between $C = 0$ and C gives:

$$\ln\left(1 - \frac{C}{C^*}\right) = k_La\,t \quad (2)$$

k_La, expressed in s^{-1}, was then estimated as the slope of the straight line obtained by plotting the experimental data of $\ln(1 - C/C^*)$ vs. time.

2.5 Correlation of k_La with Operating Variables

Several equations are available in the literature to correlate k_La to different operating variables. According to Badino et al. [23], they can be gathered in two main groups: those that do not make use of any dimensional criterion and those based on dimensional analysis. The latter have a number of advantages, one of which is the fact that the correlations obtained for a system can be used to estimate k_La in other systems with different sizes.

It is well accepted that the general equation which correlates k_La with volumetric power input ($P_g\,V^{-1}$) and superficial gas velocity (V_s) [24], may be simplified by equating the former term to the so-called power number (N^3D^2), thus resulting in the equation [25, 26]:

$$k_La = A(N^3D^2)^{\alpha}[V_s]^{\beta} \quad (3)$$

where N (s^{-1}) is the impeller speed, D is the impeller diameter (m), A is a constant, and α and β are empirical exponents.

Intermediate values of V_s (1.0 vvm) and N (200 rpm) were utilized in the logarithmic form of Eq. (3) to estimate the values of α and β.

This approach was selected because of its advantage in eliminating fluctuation and imprecision in measuring stirring power input delivered to the agitator [22, 27].

2.6 Standard Curve of Surfactin

First, surfactin from *Bacillus subtilis* (catalog number S3523, Sigma Aldrich, São Paulo, Brazil) was diluted with distilled water up to a concentration of 1.0 g L^{-1}. Then, 2 mL of each medium were transferred to twelve 5-mL test tubes, in six of which toluene had been added up to a concentration of 3 vol %. The above concentrated surfactin solution was then added at different concentrations (5, 10, 50, 100, 200, and 500 µg L^{-1}) either in toluene-free or toluene-containing media. Samples were finally placed in a shaker incubator, model G-25KC (New Brunswick Scientific, Edison, NJ, USA), at 30 °C and 200 rpm, and surface tension was measured at both the start and the end (48 h) of each test.

2.7 Statistical Analysis

k_La determinations were carried out in duplicate, while those of surface tension in triplicate, and the results expressed as mean values. Mean values were submitted to analysis of variance (ANOVA) by the Statistica Software 6.0 (StatSoft Inc, Tulsa, OK, USA). They were compared using Tukey's test at significance level $P < 0.05$. Different letters were used to label values with statistically significant differences among them.

3 Results and Discussion

3.1 Volumetric Oxygen Mass Transfer Coefficient

The volumetric oxygen mass transfer coefficient (k_La) is the most important parameter for any aerobic bioprocess. This parameter is influenced by a number of factors such as agitation intensity, aeration, type and features of the bioreactor, composition of culture media, and type and concentration of microorganism [10–14]. In turn, oxygen mass transfer plays a fundamental role in the control of oxygen available for the metabolism of aerobic microorganisms in the bioreactor.

Seeking possible application of the results of this work to bioremediation processes devoted to the removal of organic pollutants, the first part dealt with the effects of aeration and agitation on k_La in four different media with variable salt composition and osmolarity, namely, basal saline medium (BSM), low saline medium (LSM), Bushnell-Hass medium (BHM), and a medium simulating seawater (SW). The results of these tests carried out either with or without toluene as a model pollutant are summarized in Tab. 2 and compared with those obtained using glucose or yeast extract as carbon and nitrogen sources to sustain biodegradation.

As expected from general knowledge about oxygen distribution in liquids, the best operating conditions, i.e., those which ensure the highest k_La values, were obtained in all the tested media at the highest levels of both agitation (300 rpm) and aeration (1.5 vvm). These results are consistent with the well-known influence of agitation on air bubble distribution and mixing, in that insufficient agitation results in poor oxygen transfer, especially in highly viscous media [28]. Looking at k_La and osmolarity values, in general they appear to be

inversely related, even though a strict correlation cannot be established between these two parameters.

The lowest k_La values and the highest values of osmolarity were always observed in yeast extract-containing media (Tab. 2), likely due to the well-known colloidal nature and viscosity-increasing action of this component, both features that contribute to reduce O_2 solubility.

For instance, taking mean values to make comparisons easier, at 300 rpm agitation and 1.5 vvm aeration, k_La was 0.0182 s^{-1} either in non-supplemented media or in glucose-supplemented media, and even fell to 0.0125 s^{-1} in the presence of yeast extract. A less marked k_La reduction (to 0.0166 s^{-1}) was also observed in toluene-containing LSM, BHM, and SW, likely due to the hydrophophic nature of this compound. It is noteworthy that the range of k_La values obtained in this study (Tab. 2) are in agreement with those reported in the literature in different media, ranging from 0.001 to about 0.020 s^{-1} [9, 21, 25].

As expected from the much higher osmotic pressure of salts compared to organic compounds, the osmolarity increased in all cases with salt concentration being about 4, 5, and 8 times higher in SW than in BSM, BHM, and LSM, respectively.

3.2 Correlations of k_La with the Power Number and Superficial Gas Velocity

In distilled water, the values of α (0.132) and β (0.506) of Eq. (3) (Tab. 3) which represent the slopes of the straight lines that correlate $\ln k_La$ with $\ln N^3D^2$ and $\ln V_s$, respectively, fall within the very wide ranges of values reported in the literature for water and different liquid solutions ($0.1 \leq \alpha \leq 0.7$ and $0.3 \leq \beta \leq 0.8$) in differently shaped bioreactors and under quite different operating conditions [22, 28, 29]. As suggested by Garcia-Ochoa and Gomez [25], such variability may be due to different bioreactor designs, media properties, and operating conditions.

On the other hand, the values of α and β in BSM, LSM, BHM, and SW supplemented with glucose, yeast extract or toluene were statistically significantly higher than their respective values obtained in the absence of these compounds (Tab. 3). This is likely because their presence in the liquid phase altered the physicochemical properties of the bulk and then affected oxygen mass transfer [1, 14, 28]. Such increases were particularly high in the presence of toluene (α = 0.306, 0.363, 0.344, 0.237 and β = 0.968, 0.974, 1.052, 1.056 for BSM, LSM, BHM, and SW, respectively), whose hydrophobicity may have promoted bubble coalescence leading to larger bubbles and decreasing the interfacial area [1, 22].

3.3 Effects of Surfactin and Toluene on Surface Tension

To simulate the conditions which occur in bioremediation processes, the last part of this work addressed the effect of increasing concentrations of surfactin on the surface tension of the selected media, either with or without 3 vol% toluene. As one can see in Fig. 1 a, a progressive increase in surfactin concentration led to a corresponding decrease in surface tension of distilled water taken as a reference, as the likely result of micelle formation. When toluene was added as well, the interactions in the bulk became more dispersed and the surface tension decreased even more. Moreover, whereas no statistically significant variation was observed between the start and the end (48 h) of each test without toluene, in the presence of this pollutant, the final values of surface tension were always lower than the initial ones, suggesting the occurrence of mixed micelles between surfactin and toluene whose size or number increased along the time.

Following the suggestion of Abdel-Mawgoud et al. [30], who emphasized the importance of knowing the effect of salinity on the activity of biosurfactants to predict their success in bioremediation of oil-contaminated seawater, the same experimental protocol was applied to the selected salt media with the exception of BSM that had an osmolarity very close to that of BHM.

In all tested media, the presence of salts induced a generalized increase in surface tension at the end of tests compared to at the beginning, i.e., a trend opposite to that observed in water, which suggests a negative interference of salts on toluene-surfactin interaction and micelle formation.

Table 3. Estimated correlation coefficients, α and β, in Eq. (3) for water (W), basal saline medium (BSM), low saline medium (LSM), Bushnell-Hass medium (BHM), and the medium simulating seawater composition (SW). (Glu) Samples supplemented with 20 g L^{-1} glucose; (YE) samples supplemented with 50 g L^{-1} yeast extract; (Tol) samples supplemented with 3 vol% toluene. Different letters in the same column mean statistically significant difference according to the test of Tukey ($P < 0.05$).

Medium	α	β	R^2
W	0.132^a	0.506^a	0.995
BSM	0.162^b	0.606^d	0.969
BSM + Glu	0.185^d	0.622^e	0.979
BSM + YE	0.301^l	0.654^k	0.988
BSM + Tol	0.306^m	0.968^m	0.979
LSM	0.195^f	0.869^i	0.997
LSM + Glu	0.230^i	0.879^k	0.981
LSM + YE	0.245^k	0.892^l	0.985
LSM + Tol	0.363^o	0.974^m	0.989
BHM	0.173^c	0.510^b	0.966
BHM + Glu	0.200^g	0.697^g	0.998
BHM + YE	0.208^h	0.771^h	0.991
BHM + Tol	0.344^n	1.052^n	0.987
SW	0.179^d	0.545^c	0.968
SW + Glu	0.178^d	0.802^i	0.989
SW + YE	0.193^f	0.877^k	0.998
SW + Tol	0.237^j	1.056^n	0.988

A proof of this hypothesis could be the fact that such an increase was observed even in LSM, which exhibited the lowest osmolarity among the media supplemented with 3 vol % toluene (65 mOsmol L^{-1}) and that this occurred only at the highest surfactin concentrations (> 50 µg L^{-1}), but not in the absence of toluene (Fig. 1 b). On the other hand, such an increase did not take place at the lowest surfactin concentrations (5 and 10 µg L^{-1}); probably because the overall salt concentration was not enough to hinder micelle formation under these conditions.

According to its higher osmolarity either in the presence (107 mOsmol L^{-1}) or in the absence of toluene (110 mOsmol L^{-1}), BHM showed substantially higher surface tension than both the LSM and water under all the tested conditions (Fig. 1 c). For instance, at the end of tests, in the presence of 500 µg L^{-1} surfactin and 3 vol % toluene, the surface tension (35.6 dyne cm^{-1}) was approximately 55 % higher than in water. As suggested by Hanna et al. [31], the presence of salts may have favored the formation of more and larger micelles that were added vertically as a stem, encapsulating more hydrocarbon molecules. This improved aggregation capability of the surfactant that may have favored toluene dispersion and solubilization was confirmed by Zhai et al. [32], who observed, without salts, the formation of simple spherical micelles. Larger vesicles formed with salts even at lower biosurfactant concentration.

Similar to the results of this study, Tabatabaee et al. [33] observed that, without salt, the surface tension decreased throughout the runs, whereas it increased with progressively increasing salt concentration. On the contrary, Haghighat et al. [34], who assessed the effect of different NaCl concentrations (2–20 %) on biosurfactants synthesized by *Bacillus subtilis* and *Bacillus licheniformis*, found that the presence of salt did not significantly influence the surface tension, even at high concentrations (20 %).

Fig. 1 d, illustrates the behavior of surface tension in SW. Contrary to expectations, at the end of tests, SW exhibited only 8 % higher surface tension than LSM (Fig. 1 b) in the presence of 500 µg L^{-1} surfactin and 3 vol % toluene. Haghighat et al. [34] observed that surfactin activity remained almost constant up to 6 % NaCl, a salt concentration that was about twice that present in SW in the present study (approximately 3 %). Therefore, the results observed in SW (Fig. 1 d) can be ascribed to enhanced toluene solubilization due to the formation of large vesicles rather than micelles in the presence of very high salt concentration [32]. With toluene solubilization enhanced

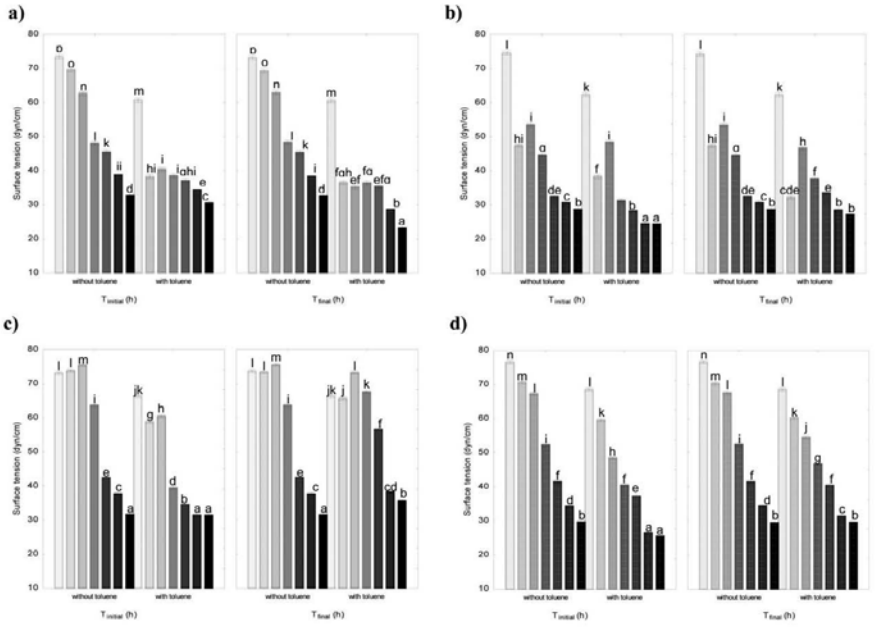

Figure 1. Surface tension in distilled water (A), low saline medium (B), Bushnell-Haas medium (C), and a medium which simulates seawater composition (D) with or without 3 vol % toluene, in the presence of surfactin at different concentrations (µg L^{-1}): □ 0; ■ 5; ■ 10; ■ 50; ■ 100; ■ 200; ■ 500. Mean values (n = 3) ± standard deviations determined at the beginning ($T_{initial}$) and the end (T_{final}) of tests. Different letters mean statistically significant differences among values in the same box (P<0.05).

by vesicles, there may have been leftover surfactin monomers that hindered the surface tension increase [35]. These results are quite reassuring in view of a potential use of this technique in bioremediation of oil-polluted marine water.

Resuming, the highest toluene removals by surfactin, corresponding to the lowest values of surface tension, occurred at the end of every test at the highest surfactin concentration (500 $\mu g\,L^{-1}$) in all tested media, specifically in a) distilled water, 23.3 dyne cm^{-1}, b) low saline medium, 24.4 dyne cm^{-1}, c) Bushnell-Haas medium, 31.4 dyne cm^{-1} and d) seawater, 25.6 dyne cm^{-1}.

4 Conclusions

In this study, the volumetric oxygen mass transfer coefficients (k_La) are compared in different saline solutions to be used as possible media for aerobic bioremediation of oil-contaminated sites, namely, basal saline medium, low saline medium, Bushnell-Haas medium, and a medium which simulates seawater composition. The best operating conditions able to ensure the maximum values of k_La were an agitation intensity of 300 rpm and an aeration rate of 1.5 vvm. The presence of salts in the media increased the osmolarity, while the surface tension decreased when surfactin was added to toluene-containing media. The addition of 3 vol % toluene as a model pollutant led to higher values of the exponents of the equation which correlates k_La to the power number (a) and the superficial velocity (β) in all tested media, compared to those obtained adding glucose or yeast extract. Among all toluene-containing media, the one which simulates seawater composition showed the highest surface tension reduction, which is a promising result in view of employing surfactin-producing microorganisms to bioremediate oil-contaminated seawater. The results obtained in this preliminary work provide a useful starting basis for future studies on the treatment of oil spills by hydrocarbon-degrading microorganisms able to release biosurfactants and suggest that optimal operating conditions should be investigated to enable efficient bioremediation.

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Symbols used

C^*	[$g\,m^{-3}$]	oxygen saturation concentration at equilibrium
dC/dt	[$g\,m^{-3}\,s^{-1}$]	rate of O_2 accumulation in the liquid phase
N	[s^{-1}]	impeller speed
D	[m]	impeller diameter
V_s	[vvm]	gas velocity
k_La	[s^{-1}]	volumetric oxygen mass transfer coefficient
a	[–]	empirical exponent
β	[–]	empirical exponent
τ	[s]	oxygen probe response time

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Chemical Engineering Technology Research Article 9

Research Article: Toluene can be removed from contaminated sites via bioremediation through the addition of biosurfactant compounds which reduce the surface tension. The volumetric oxygen mass transfer coefficients are compared in different saline solutions to be used as possible media for aerobic bioremediation of oil-contaminated sites.

Volumetric Oxygen Mass Transfer Coefficient and Surface Tension in Simulated Salt Bioremediation Media

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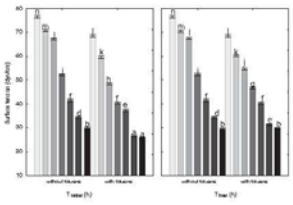

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Influence of toluene and salinity on biosurfactant production by Bacillus sp.: scale up from flasks to bench-scale bioreactor

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Influence of toluene and salinity on biosurfactant production by *Bacillus* sp.: scale up from flasks to bench-scale bioreactor

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ABSTRACT

To select the best biosurfactant producer, *Pseudomonas putida*, *Bacillus megatherium*, *Bacillus licheniformis* and *Bacillus subtilis* were cultured in flasks on media with different salinity [low salinity (LS), Bushnell-Haas (BH) and artificial sea water (SW) media] supplemented or not with toluene as a model pollutant. Toluene inhibited the growth of all microorganisms and stimulated the biosurfactant production. *B. subtilis* exhibited the best performance being able to lower the surface tension (ST) in the LS medium to 65.5 mN/m in the absence of toluene, and to 46.5 mN/m in the BH medium in presence of toluene, corresponding to ST reductions of 13.0 and 27.5 mN/m, respectively. Scaling up the process to a bench-scale fermentor, the best results were obtained in the LS medium, where *B. subtilis* was able to reduce toluene concentration from 26.0 to 4.3 g/L within 12 h and ST by 17.2 mN/m within 18 h. The results of this study point out that *B. subtilis* is an interesting biosurfactant producer, which could be used in the bioremediation of toluene-contaminated water.

Keywords: Bioreactor; Toluene; Biosurfactant; Saline media; *Bacillus subtilis*; Bioremediation.

1 Introduction

Concerns related to the potential contamination of soil and water by oil and its derivatives has been gradually increasing. The main sources of contamination are: a) unintended spill during the transportation of fuels by ships and trucks; b) leaking from underground storage tanks such as those used in gas stations, which are subject to corrosion; c) oil extraction and processing operation; and d) inadequate disposal of oily wastes from industries that use oil derivatives to produce plastics, solvents, pharmaceuticals, cosmetics, etc. (Du *et al.*, 2014; Kavitha *et al.*, 2014).

Residues produced by such activities are composed of oils, fats, organic compounds and metals. Among the organic compounds, there are alkanes, cycloalkanes, benzene, toluene, xylenes, phenol and polycyclic aromatic hydrocarbons (Di Martino *et al.*, 2012). Many of these compounds are considered extremely polluting because, besides being toxic, some of them have carcinogenic and mutagenic potential to human beings (Janbandhu and Fulekar, 2011).

The sea environment has been suffering constant oil spills, and the release of thousands of tons of petroleum contaminating the ocean has been constantly reported (Goldman *et al.*, 2015). In Brazil, for example, one of the most impactful spills took place in November 2011, when 594.3 thousand liters of petroleum were released from the Sedco 706 platform, operated by Chevron in Campos Basin (Rio de Janeiro), creating a polluted area of about 163 km² (Souza, 2013).

Gasoline and diesel contain hydrocarbons from the BTEX group (benzene, toluene, ethylbenzene and xylenes), which are the first ones to reach groundwater (Mariano *et al.*, 2007). Consequently, such highly-toxic compounds (except ethylbenzene) are the major causes of death related to drinking water contamination (Di

Martino *et al.*, 2012). Fellenberg (1980) reported that petroleum and its derivatives, when in contact with water, spread creating a thin film on the surface that prevents not only gas exchange between air and water, but also light from reaching phytoplankton, thus affecting the food chain. Accordingly, Asimiea and Sam-Wobo (2011) observed a strong impact of such compounds on phytoplankton communities, which suffered structural changes. The potential threat of hydrocarbons to human health is linked to both their physical and chemical properties, which allow them to be absorbed by the skin and quickly spread throughout the organism (Costa *et al.*, 2012).

Toluene has moderate systemic toxicity to humans. When ingested, it is completely absorbed by the gastrointestinal tract, quickly spreads through the body, especially the fatty tissues, and is metabolized and excreted through the urine. When inhaled, toluene affects the central nervous system (Fellenberg, 1980). In addition, embryotoxic and fetotoxic effects have been observed, though there is no clear evidence of teratogenic or carcinogenic activity in laboratory animals or humans (WHO, 2006; Zhang *et al.*, 2013).

In light of the problems caused by hydrocarbons to both the environment and human beings, the search for methods able to remove such compounds or reduce their occurrence in the environment has greatly increased in the last years. The methods used to achieve this goal are based on chemical, physical or biological means. Due to the high cost of physical treatments, and to the fact that chemical methods release substances that are harmful to the local biota, research efforts have been mainly focused on developing sustainable technologies using natural biodegradable compounds (Lin *et al.*, 2012).

Bioremediation processes, based on the use of living organisms to clean-up petroleum-hydrocarbon contaminated sites, consist in the biological decomposition of such organic pollutants by microorganisms that use them as carbon source to get energy, thereby releasing carbon dioxide, water, mineral salts and other gases (Banat *et al.*, 2010). According to García *et al.* (2006), biodegradation of organic pollutants in the presence of surfactants happens when microorganisms use them as carbon and energy source. In this process, there is, firstly, the breakdown of hydrocarbon chains, which promotes a structural modification and immediate loss of amphipathicity. Then, the products of this first degradation are converted into CO_2, water and minerals. The formation and accumulation of biosurfactants are related to a series of parameters that, according to Mukherjee *et al.* (2006), need further optimization in order to reach higher yields and lower costs. The promising future of biosurfactants depends on the use of low-cost raw materials and optimization of growth conditions to increase their production (Cortés-Camargo *et al.*, 2016).

In this context, the aim of this work was to investigate the ability of biosurfactants released during cultivations of *Bacillus subtilis, Bacillus megatherium, Bacillus licheniformis* and *Pseudomonas putida* to promote toluene uptake in three aqueous media with different salinity, namely a low salinity medium, the Bushnell-Haas medium and a medium simulating seawater composition. To this purpose, the biosurfactant ability to lower surface tension was evaluated. The process was finally scaled up to a bench-scale fermentor to evaluate the influence of salinity on the ability of the best biosurfactant producer, *B. subtilis*, to remove toluene.

2 Materials and Methods

2.1 Growth media

The Bushnell-Haas medium (BH), with osmolarity of 110 mOsmol/L and surface tension (ST) of 74.0 mN/m, had the following composition (mg/L): calcium chloride (23.0), magnesium sulfate (20.0), monobasic potassium phosphate (61.0), dibasic potassium phosphate (55.0), ammonium nitrate (50.0) and ferric chloride (69.0). The low salinity medium (LS), with osmolarity of 11 mOsmol/L and ST of 78.5 mN/m, was prepared by 1:10 (v/v) dilution of the BH medium with distilled water. The medium simulating seawater composition (SW), with osmolarity of 1,100 mOsmol/L and ST of 74.5 mN/m, was prepared pursuant to the adapted formulation of Bidwell and Spotte (1985) and had the following composition (mg/L): calcium chloride (27.6), magnesium sulfate (6.9), magnesium chloride (5.4), calcium chloride (1.4), potassium chloride (0.6), sodium bicarbonate (0.2), potassium bromide (0.027), strontium chloride (0.02), manganese sulfate (0.01), sodium phosphate (0.01) and sodium molybdate (0.001).

All these saline media were supplemented or not with 3.0 % (v/v) toluene, corresponding to a starting toluene concentration of 26.0 g/L.

2.2 Microorganisms

The strains *Bacillus subtilis* ATCC 6051, *Bacillus megatherium* ATCC 14581 and *Bacillus licheniformis* ATCC 14580 were tested for their ability to uptake and degrade toluene in aqueous media, while *Pseudomonas putida* ATCC 12633 was selected as a reference strain for comparative purposes. All the strains were acquired from André Tosello Foundation (Campinas, SP, Brazil) and stored until use at -80 °C in the BH medium containing 5% (w/v) yeast extract and 50% (v/v) glycerol at pH 7.0.

2.3 Bacteria growth in shake flasks

Pre-cultures were prepared transferring 1.0 mL of each strain suspension into 100 mL-flasks containing 15 mL of BH medium with 5% (w/v) of yeast extract and incubating them at 30 ºC and 200 rpm in rotary shaker, model G-25KC (New Brunswick, Enfield, CT, USA) for 24 h. Two mL of pre-cultures, corresponding to a cell concentration of about 0.05 g/L, were used to inoculate test tubes containing 20 mL of each saline medium and used in tests performed in shaker at 200 rpm and 30 ºC for 96 h. The whole content of three test tubes was used for each microorganism after 48, 72 and 96 h to determine biosurfactant and biomass concentrations. Runs were carried out in triplicate, and the results expressed as mean values ± standard deviations.

Controls were carried out with the same culture broths without inoculum in order to evaluate toluene loss by volatilization. In all cases studied, more than 95% of initial toluene remained at the end of the process, thus eliminating the possible effect of toluene volatilization under the selected conditions.

2.4 *B. subtilis* cultivation in bioreactor

B. subtilis, which showed the best performance in terms of ST reduction in flasks, was selected to perform cultivations in a 3-L bench-scale bioreactor, model BioFlo®/Celligen® 115 (New Brunswick, Enfield, CT, USA), containing 1.5 L of the saline medium inoculated with 30 mL of microbial suspension and supplemented with 3.0 (v/v) toluene, corresponding to a starting concentration of 26.0 g/L. Volatilization of toluene was minimized by using a glass cooler at the exit of the bioreactor (10 °C), while temperature inside the bioreactor was maintained at 30 °C by recirculating water

from a water bath, model Alpha RA 8 (Lauda, Delran, NJ, USA), into an external jacket. Pre-cultures were harvested under similar conditions as earlier described. All the cultivations were carried out in triplicate at 30°C, 200 rpm and 1.0 vvm aeration for 42 h, and the results expressed as mean values ± standard deviations. Samples were taken at different times to determine ST and cell concentration. Material balances were made for each run resorting to a CO_2 electrode, model InPro 5000 (Mettler-Toledo, Columbus, OH, USA), and their range of variation was 94.7 to 96.4%.

2.5 Analytical procedures

Biomass concentration along the runs was determined by optical density (OD) measurements at 560 nm and expressed as dry cell concentration using a calibration curve. Samples (5.0 mL) were filtered through pre-dried and weighted membrane filters with 0.45 μm (Millipore, Billerica, MA) and then dried in an oven at 100 °C for 24 h.

Since the biosurfactant produced by the selected microorganisms had not been yet identified, no experimental methodology was available to detect its concentration directly. Therefore, the surface tension (ST) decrease along the runs was assumed to be directly proportional to the biosurfactant concentration. ST of culture media was measured according to the Ring method described by Kim *et al.* (2000) using a tensiometer, model K9-Mk1 (Krüss, Hamburg, Germany), at 25 ± 1 °C and expressed in mN/m.

As far as the quantification of toluene is concerned, at first it was extracted using a 1:3 (v/v) sample:chloroform ratio. The chloroform extract (0.5 μL) was then injected into a gas chromatograph, model 7820A (Agilent Technologies, Santa Clara, CA, USA), equipped with a HP-5 column containing 5% of phenyl methyl silicone (30 m x

0.32 mm) with film width of 0.25 μm (Agilent Technologies) and a flame ionization detector. Chromatographic analyses were carried out at initial temperature of 40°C for 1 min, heating at 10 °C/min up to 80 °C, and keeping the system at this temperature for 1 min. Helium was used as carrier gas at flowrate of 28.5 mL/min.

The shape of all microorganisms was observed either at the beginning or the end of cultivations by an optic confocal microscope, model MRC-1024 (Bio-rad, Munich, Germany).

3 Results and Discussion

3.1 Growth of microorganisms in flasks and biosurfactant production

Preliminary runs were performed using a rotary shaker to select, among different *Bacillus* species (namely *B. subtilis*, *B. megatherium* and *B. licheniformis*), the best biosurfactant producer in terms of surface tension reduction (ST_{red}). Bacteria were grown in media with different salinities either with or without toluene, while *P. putida* was tested as a control microorganism for comparison purposes, taking into account that it has been thoroughly studied just because of its high biosurfactant production capacity (Aparna *et al.*, 2011). Biomass concentration of the selected microorganisms in the media employed for this study varied widely either in the absence (0.11 ± 0.01 g/L to 1.32 ± 0.01 g/L) (Table 1) or in the presence (0.08 ± 0.01 g/L to 1.02 ± 0.01 g/L) (Table 2) of toluene. Microscopic examinations made both at the beginning and the end of cultivations did not reveal any apparent morphological change in any of the microorganisms employed in this study.

In the low salinity (LS) medium without toluene, *B. licheniformis* grew better than all the other microorganisms, reaching a biomass concentration (0.82 ± 0.02 g/L) after 96 h of cultivation even higher than the control (*P. putida*) (0.36 ± 0.02 g/L) (Table 1). On the other hand, the presence of toluene was detrimental for all the selected microorganisms but the control, in that it reduced considerably their growth. As a matter of fact, cell concentration of *B. subtilis*, which was the microorganism that showed the fastest growth, did not exceed 0.16 ± 0.02 g/L after 72 h (Table 2).

As far as the Bushnell-Haas (BH) medium is concerned, its intermediate salinity seemed to stimulate the growth of all the microorganisms under almost all the conditions tested compared to the LS one. In the absence of toluene, *B. licheniformis* showed again the highest growth among the tested bacilli, achieving a biomass concentration after only 48 h as high as 0.62 ± 0.03 g/L (Table 1), while in the presence of toluene the highest growth was obtained with *B. megatherium* after the same time (0.52 ± 0.01 g/L) (Table 2). Nonetheless, biomass concentration was remarkably reduced at longer times either in the presence or in the absence of toluene.

Using the medium simulating seawater (SW) composition, i.e. that with the highest salinity, *B. subtilis* and *B. licheniformis* reached the highest biomass concentrations in the absence of toluene (0.80 ± 0.03 g/L and 0.76 ± 0.01 g/L, respectively) but after longer time (72 h) compared to the BH medium, which suggests that these microorganisms require a long adaptation period in the presence of high salt levels. In the same medium supplemented with toluene (Table 2), *B. subtilis* showed more stable growth compared with the other bacilli, with an average cell concentration of 0.38 g/L during the whole run, thus behaving as the strain more resistant to highly salty conditions.

As regard the capacity of the selected microorganisms to produce biosurfactants, Fig. 1 illustrates their ability to reduce surface tension either with or without toluene, while the corresponding values of ST_{red} are listed in Tables 1 and 2.

It is usually assumed that a compound has to be able to reduce ST by at least 8 mN/m to be considered a surfactant (Van der Vegt *et al.*, 1991). As shown in Table 1, in the absence of toluene, *B. subtilis* was able to reduce ST of the LS medium by 8.4 ± 0.5 mN/m after 72 h and that of the SW medium by 13.0 ± 0.1 mN/m after 96 h. Reducing ST by 11.8 ± 0.3 mN/m after 48 h, *B. licheniformis* behaved even better than *B. subtilis* in the LS medium but not in the SW one, whereas *B. megatherium* was disappointing in all the culture media tested, reducing the surface tension by a maximum of 5.5 ± 0.3 mN/m in the BH medium.

To select the best microorganism to be used in future bioremediation applications, we tried to make an overall comparison among the ST_{red} skills of the selected bacilli. The results of Table 1 show that, in spite of the poor results in terms either of cell concentration or ST_{red} in the LS medium without toluene, *B. subtilis* was capable of reducing ST more than *B. megatherium* and *B. licheniformis* in the other media. According to Desai and Banat (1997), efficient biosurfactants have low critical micelle concentration (CMC), i.e. a small quantity of surfactant is needed to reduce ST, which suggests that the biosurfactant produced by *B. subtilis* could have been more effective than those produced by the other species. Conversely, *P. putida* (used as control for comparison) was the microorganism that showed the best cell growth and that mostly reduced ST in almost all the systems without toluene. ST reduction was the highest in the LS medium after 72 h (21.2 ± 1.1 mN/m) and decreased almost

proportionally to the increase in salinity (16.4 ± 0.5 mN/m in the BH and 11.8 ± 0.3 mN/m in the SW media, respectively).

But the most interesting finding is that the use of toluene as a carbon source significantly stimulated the short-term production of biosurfactants at the expense of growth. Regardless of the salinity level of media, all microorganisms tested did in fact ensure ST reductions greater than 19 mN/m within only 48 h. Our general conclusion is that toluene may have enhanced biosurfactant production, hence indirectly enhancing non-specific permeability of the cytoplasmic membrane and resulting in ATP leakage (Rodrigues *et al.*, 2006).

The best performance was observed again with the control, with a ST reduction by 33.6 ± 0.4 mN/m in the BH medium. However, such a reduction considerably decreased with increasing the fermentation time. Particularly it stands by what happened in the LS medium where ST reduction decreased from 25.8 ± 0.9 mN/m after 48 h to only 0.8 ± 0.3 mN/m after 96 h. These values are in fair agreement with those of Haghighat *et al.* (2008), who reported for *B. licheniformis* and *B. subtilis* cultivated in rotary shaker on raw oil (2%) as carbon source, under the same conditions as the present study, ST reductions by 30 and 29 mN/m, respectively, after 48 h of growth. However, the raw oil employed by these authors comprised several different carbon sources, while toluene was the only source used in this study besides yeast extract.

Mnif *et al.* (2001), who used petroleum-derived hydrocarbons, reported that the tested strains, among which a *B. licheniformis* strain isolated from oilfields, did not show any capacity of using toluene as a carbon source. Consistently, Nicholson and Fathepure (2004) observed that only 0.004% of toluene was consumed along a 7 days-run in rotary shaker, while the same concentration of benzene, ethylbenzene and xylene

needed approximately 15 days to be metabolized. These observations are consistent with the well-known harmful effects and great difficulty to remove toluene from the environment.

3.2 Production of biosurfactant in bench-scale bioreactor

Based on the results of previous experiments performed in rotary shaker, which demonstrated that an organic carbon source like toluene can stimulate the production of biosurfactants, *B. subtilis* was selected to scale up the process to a bench-scale bioreactor. Several researches studying to bioremediation of aqueous media contaminated by hydrocarbons reported starting ST values close or higher than 65 mN/m, and a subsequent reduction to less than 35 mN/m depending on the capacity of bacteria to produce biosurfactants (Haghighat *et al.*, 2008; Nicholson and Fathepure, 2004; Xia *et al.*, 2011).

As is shown in Fig. 2A, illustrating the growth of *B. subtilis* in the LS medium, ST was initially 73.5 mN/m, remained almost constant for about 12 h, then decreased simultaneously with the microbial growth, and was reduced by 17.2 mN/m after 15 h. Biomass continued to grow until 24 h reaching a maximum cell concentration of 0.36 g/L, despite the concentration of toluene in the medium decreased from 26.0 to only 4.3 g/L after 12 h. Such a low biomass yield could have been due to the use of non-adapted cells in addition to the same energy-consuming biosurfactant production. The inoculum was in fact prepared in a medium where yeast extract was the only carbon source, while subsequent runs were done under different conditions, i.e. variable salinity and presence of toluene as additional carbon source in this run. ST kept almost constant between 18 and 38 h likely due to the achievement of biosurfactant CMC during the exponential

phase of *B. subtilis* growth; in fact, as suggested by several authors, when micelle formation starts, ST in the fluid does not vary (Desai and Banat, 1997; Soberón-Chávez and Maier, 2010).

Recently, Vaz *et al.* (2012), after observing a significant decrease in ST from 4 to 24 h of fermentation, when the lowest value of ST (30.1 mN/m) was reached, concluded that biosurfactant production is associated to growth. The authors came to this conclusion. Likewise, a decrease in ST from 71.2 to 27 mN/m was observed by Xia *et al.* (2011) with *B. subtilis* just at the beginning of the run, thus confirming a growth-associated production of biosurfactant.

In the present study, after 24 h cell concentration decreased from 0.36 to 0.16 g/L at the end of the run (Fig. 2A). Considering that more than 84% of toluene was consumed within the first 15 h, the only alternative carbon source for the microorganism might have been the produced biosurfactant itself (García *et al.*, 2006), as confirmed by the final ST increase in the LS medium up to 61.3 mN/m.

Runs in the BH medium (Fig. 2B) showed results a little different from those obtained in the LS medium (Fig. 2A). Biomass concentration was in fact higher (0.90 g/L), and, consequently, ST was progressively reduced up to the end of the run. Regarding the SW medium (Fig. 2C), ST decreased from 73.1 mN/m at the start to 67.1 mN/m after 15 h, i.e. to a value that was practically the same as those obtained in the BH and LS media (Figs. 2A and 2B).

In the SW medium (Fig. 2C), toluene concentration remained practically constant (26.0 – 24.1 g/L) during the first 12 h, and then suddenly decreased to 0.11 g/L up to 15 h, a period during which biosurfactant was produced. These results confirm that *B. subtilis* utilized toluene as primary carbon source to sustain both its growth and

biosurfactant production. Many authors supported the extracellular nature of biosurfactants produced by *Bacillus* sp. (Desai and Banat, 1997; Aparna *et al.*, 2011; Cortés-Camargo *et al.*, 2016). Consistently, Pinto *et al.* (2009) observed that there was no significant difference between ST values of the medium with (58.7 mN/m) or without (56.7 mN/m) *B. subtilis* cells, hence concluding that biosurfactant was extracellularly released.

Fig. 3A shows that foam formation by *B. subtilis* cultures in the LS medium started after about 12 h, remarkably increased after 24 h and continued until the end of the run (42 h). The behavior was qualitatively similar in the BH medium, but the production of foam was so pronounced that it occupied the bioreactor almost completely (Fig. 3B). Similarly, Chen *et al.* (2006) observed foam formation after 12 h with *B. subtilis* grown in M9 medium with 0.2% glucose. According to Winterburn and Martin (2012), the operating conditions of the fermentor such as aeration and stirring rate have a strong influence on foam formation, in that, foam tends to accumulate on the surface of the growth medium, but, if there is enough aeration or stirring rate, it will blend back and return to the medium.

Regarding the SW medium (Fig. 3C), foam production was lower than in the LS and BH media, as the likely result of its high salt content (osmolarity of 1,100 mOsm/L) and low ST reduction capability. According to Hanna *et al.* (2005), the presence of salt in the solution would in fact be responsible for a decrease in the electrostatic repulsive forces and allow for the approximation of the polar part, thus favoring the biosurfactant micellization vertically, like a highly flexible and endless rod, with micelle agglomeration. This rod with a higher number of biosurfactant monomers was described by Zhai *et al.* (2006) as a vesicle.

Chen *et al.* (2006) observed that, after 14 h of growth, the concentration of the biosurfactant produced by *B. subtilis* was 32.5 mg/L in the M9 medium and 1.79 mg/L in the foam; therefore, they concluded that, when biosurfactant concentration exceeds CMC, foam formation begins, and micelles tend to accumulate acting as a surfactant reservoir. Consistently with these observations, in our work, CMC was reached after approximately 18 h, and foam formation appeared little after (24 h). Foam formation is important in fermentation processes devoted to the production of extracellular biosurfactants; in fact, acting as a reservoir, foam makes biosurfactant recovery easier (Junker, 2007).

Conclusions

Among the microorganisms belonging to the *Bacillus* genus used in this work, *B. subtilis* proved to be the most effective producer of biosurfactant in the selected media, namely low saline medium, BH medium and a medium simulating seawater composition. The presence of toluene in the media enhanced the production of biosurfactants at the expense of microbial growth. During fermentation, the likely formation of large vesicles rather than simple micelles could have promoted the breakdown of hydrocarbon molecules and the quick consumption of toluene as the main carbon source. The results obtained in this study suggest that *B. subtilis* could be an interesting microorganism to be used in the bioremediation of toluene-contaminated waters, although further process optimizations are required.

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Caption of figures

Figure 1. Surface tension values obtained using different culture media [LS medium (A), BH medium (B) and SW medium (C)] at different fermentation times (48, 72 and 96 h), in the absence or the presence of toluene. Biosurfactant producers: *Bacillus licheniformis* (□), *Bacillus megatherium* (○), *Bacillus subtilis* (◇) and *Pseudomonas putida* (△). Mean values (n = 3) ± standard deviations. Different letters in the same column mean that values significantly differ among them ($P < 0.05$).

Figure 2. *Bacillus subtilis* cultivations in bench-scale bioreactor in the presence of toluene. Surface tension (♦), toluene concentration (■), and biomass concentration (▲) in LS (A), BH (B) and SW (C) media.

Figure 3. Foam formation in bench-scale fermentor during cultivations of *Bacillus subtilis*. LS (A), BH (B) and SW (C) media in the presence of toluene.

Table 1. Microbial growth of different strains in low salinity (LS), Bushnell-Haas (BH) and artificial sea water (SW) media in the absence of toluene.

Medium		LS		BH		SW	
Strain	Time (h)	Biomass concentration (g/L)	ST_{red} (mN/m)	Biomass concentration (g/L)	ST_{red} (mN/m)	Biomass concentration (g/L)	ST_{red} (mN/m)
B. subtilis	48	0.11 ± 0.01^{a}	5.7±0.4	0.20 ± 0.02^{f}	3.5±0.5	0.52 ± 0.03^{i}	3.6±0.3
	72	0.24 ± 0.01^{e}	8.4±0.5	0.28 ± 0.01^{i}	4.4±0.5	0.80 ± 0.03^{n}	4.4±0.5
	96	0.24 ± 0.02^{e}	3.0±0.6	0.26 ± 0.01^{h}	0.6±0.6	0.40 ± 0.03^{f}	13.0±0.1
B. licheniformis	48	0.20 ± 0.02^{d}	11.8±0.3	0.60 ± 0.03^{p}	0.3±0.3	0.28 ± 0.02^{b}	0.8±0.2
	72	0.56 ± 0.01^{j}	2.1±1.4	0.62 ± 0.03^{q}	2.6±0.3	0.76 ± 0.01^{m}	0.4±0.3
	96	0.82 ± 0.02^{k}	2.2±1.1	0.35 ± 0.02^{j}	0.6±0.3	0.28 ± 0.02^{b}	2.8±0.3
B. megatherium	48	0.28 ± 0.02^{f}	0.1±0.1	0.48 ± 0.01^{n}	5.6±0.3	0.20 ± 0.02^{a}	2.6±0.5
	72	0.26 ± 0.03^{ef}	4.7±0.4	0.60 ± 0.02^{p}	2.4±0.5	0.44 ± 0.02^{h}	1.7±0.4
	96	0.34 ± 0.02^{gh}	1.6±0.5	0.32 ± 0.01^{k}	2.6±0.54	0.32 ± 0.03^{d}	1.6±0.6
P. putida	48	0.32 ± 0.01^{g}	12.4±0.8	0.60 ± 0.02^{p}	16.4±0.5	1.32 ± 0.01^{q}	7.6±0.3
	72	0.32 ± 0.01^{g}	21.2±1.1	1.01 ± 0.02^{r}	9.6±0.5	0.62 ± 0.01^{k}	11.8±0.3
	96	0.36 ± 0.02^{h}	7.2±1.1	0.36 ± 0.02^{j}	12.4±0.4	0.72 ± 0.03^{l}	3.8±0.3

ST_{red}: surface tension reduction

Table 2. Microbial growth of different strains in low salinity (LS), Bushnell-Haas (BH) and artificial sea water (SW) media in the presence of toluene.

Medium		LS		BH		SW	
Strain	Time (h)	Biomass concentration (g/L)	ST_{red} (mN/m)	Biomass concentration (g/L)	ST_{red} (mN/m)	Biomass concentration (g/L)	ST_{red} (mN/m)
B. subtilis	48	0.16 ± 0.02^{c}	23.5±0.7	0.08 ± 0.01^{a}	20.5±0.7	0.34 ± 0.03^{e}	21.7±0.4
	72	0.16 ± 0.01^{c}	23.5±0.7	0.14 ± 0.01^{c}	27.3±0.3	0.40 ± 0.02^{f}	20.6±0.3
	96	0.11 ± 0.01^{a}	22.3±0.5	0.10 ± 0.01^{b}	27.5±0.5	0.40 ± 0.02^{f}	23.4±0.3
B. licheniformis	48	0.11 ± 0.02^{a}	24.6±0.6	0.46 ± 0.03^{m}	24.5±0.6	0.28 ± 0.02^{b}	19.7±0.3
	72	0.12 ± 0.03^{ab}	23.3±0.5	0.26 ± 0.02^{h}	24.5±0.5	0.30 ± 0.01^{c}	19.7±0.4
	96	0.11 ± 0.02^{a}	22.2±1.1	0.16 ± 0.03^{d}	12.6±0.3	0.28 ± 0.01^{b}	17.6±0.6
B. megatherium	48	0.14 ± 0.03^{bc}	19.5±0.6	0.52 ± 0.01^{o}	20.5±0.6	0.30 ± 0.02^{c}	20.7±0.3
	72	0.12 ± 0.03^{ab}	25.6±0.6	0.22 ± 0.01^{g}	20.6±0.5	0.54 ± 0.02^{j}	19.9±0.1
	96	0.14 ± 0.02^{bc}	18.8±0.4	0.08 ± 0.02^{a}	17.6±0.5	0.28 ± 0.03^{b}	17.0±0.2
P. putida	48	0.48 ± 0.02^{i}	25.8±0.9	0.35 ± 0.03^{j}	33.6±0.4	1.02 ± 0.01^{p}	25.4±0.2
	72	0.12 ± 0.02^{ab}	15.1±1.2	0.30 ± 0.03^{i}	23.7±0.4	0.42 ± 0.01^{g}	21.7±0.4
	96	0.28 ± 0.01^{f}	0.8±0.3	0.18 ± 0.01^{e}	21.5±0.6	0.82 ± 0.02^{o}	22.0±0.3

ST_{red}: surface tension reduction

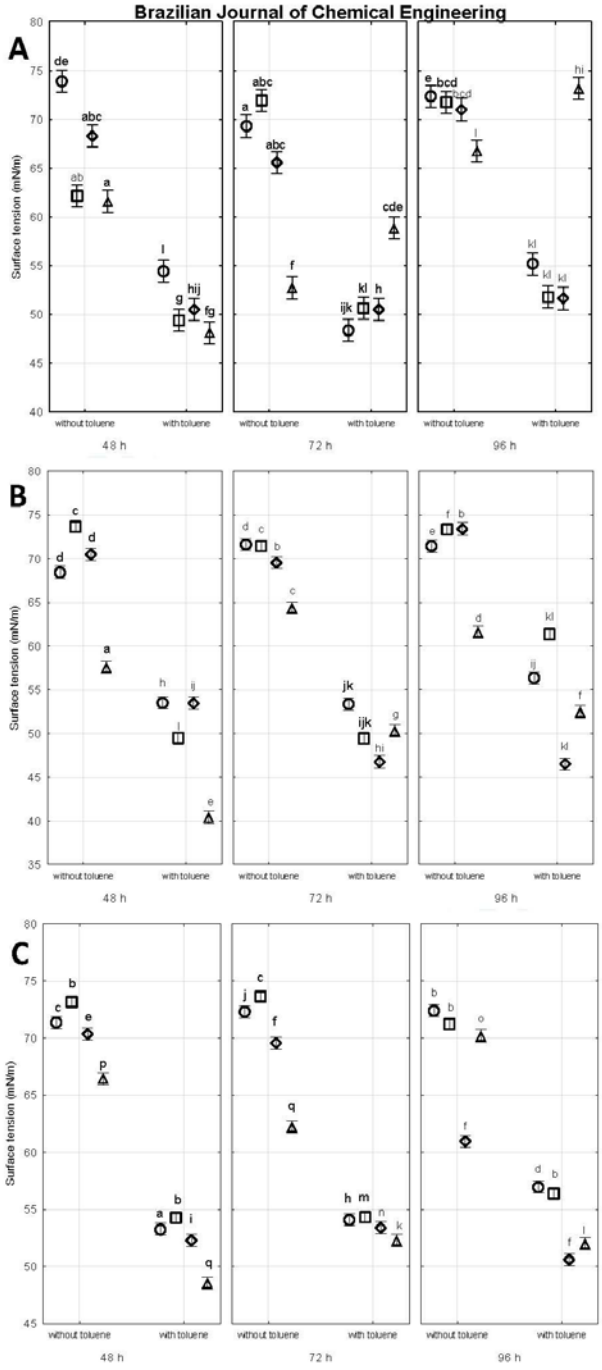
A
B
C
Surface tension (mN/m)
without toluene
with toluene
48 h
72 h
96 h

Figure 1

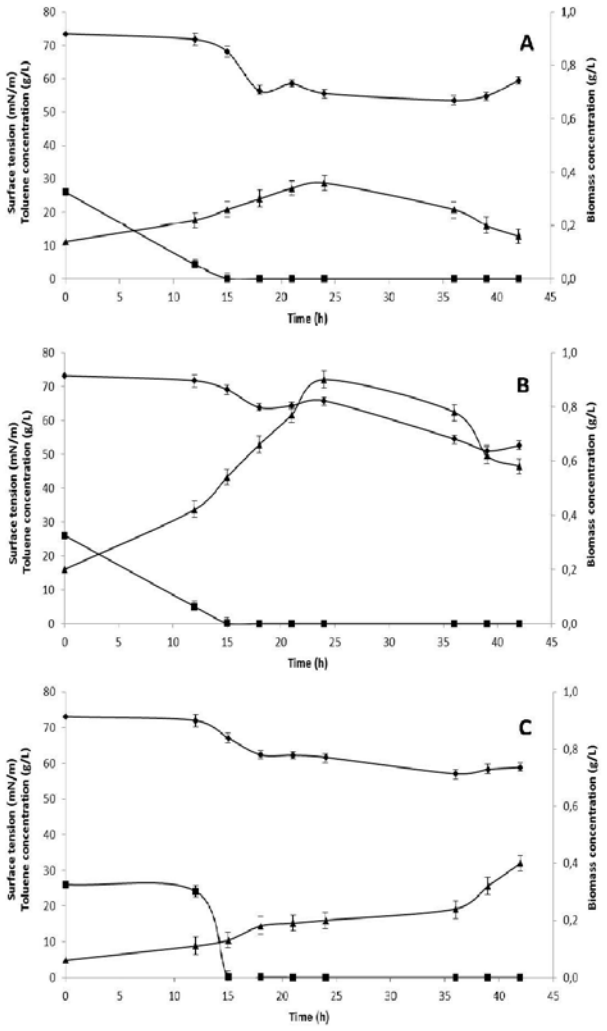
A
B
C
Surface tension (mN/m)
Toluene concentration (g/L)
Biomass concentration (g/L)
Time (h)

Figure 2

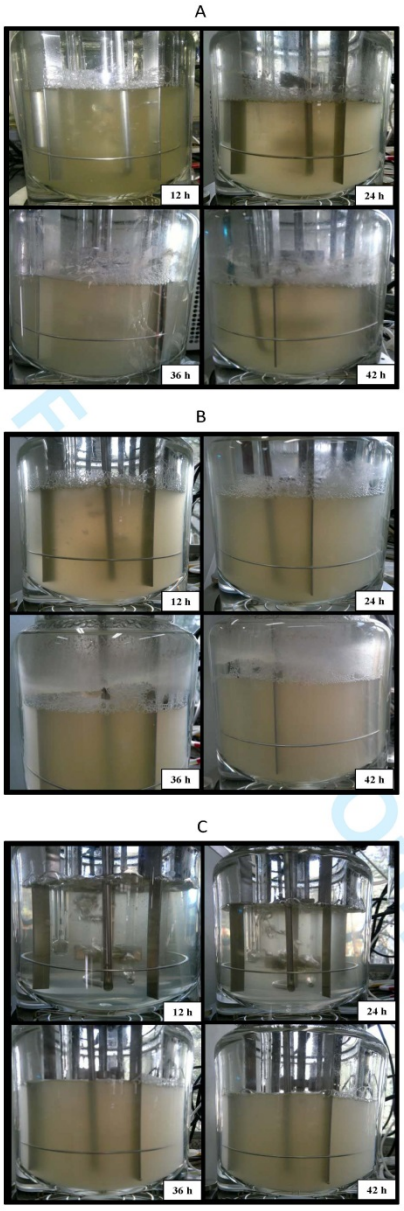

Figure 3

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Influence of temperature and pH on the production of biosurfactant, bacteriocin and lactic acid by *Lactococcus lactis* CECT-4434

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ABSTRACT

Operational conditions such as temperature and pH are well known to influence the productions of biosurfactants, bacteriocins, and lactic acid by lactic acid bacteria. The bacterial strain *Lactococcus lactis* subsp. *lactis* CECT-4434 was used to investigate the effects of temperature (30°C, 37°C and 40°C) and pH control on the production of these biomolecules, in order to establish whether they are able to preferentially address its metabolism towards one product or another. It was observed that the pH control within the range 5.0–5.3 favored the production of lactic acid, which was 38% higher than that obtained without control. The bacteriocin production was higher in the temperature range between 30°C and 37°C, evidenced by the formation of inhibition zones against *Lactobacillus sakei* and *Staphylococcus aureus*, 5–22% and 4–14% higher, respectively, compared with those achieved at 40°C. Finally, the best results for biosurfactant synthesis occurred at 37°C without pH control, with a surface tension reduction between 22.5 and 24.7 mN/m after 6–24 h of culture, respectively.

RESUMEN

Es bien conocido que determinadas condiciones operacionales, tales como la temperatura y el pH, influyen en la producción de biosurfactantes, bacteriocinas y ácido láctico por parte de las bacterias ácido lácticas. En este trabajo se empleó la cepa bacteriana Lactococcus lactis subsp. lactis CECT-4434 para investigar los efectos de la temperatura (30, 37 y 40°C) y el control del pH en la producción de estas biomoléculas, con el fin de determinar como influyen en su metabolismo. Se observó que a pH controlado, dentro del rango 5.0–5.3, se favorecía la producción de ácido láctico, que resultó 38% superior a la obtenida sin control. La producción de bacteriocina fue mayor dentro del rango de temperatura 30–37 °C, evidenciada por la formación de zonas de inhibición frente a las cepas indicadoras Lactobacillus sakei y Staphylococcus aureus, 5–22% y 4–14% respectivamente superiores que aquellos valores alcanzados a 40°C. Finalmente, se observaron los mejores resultados para la síntesis de biosurfactantes a 37 °C sin control de pH, con una reducción de la tensión superficial entre 22,5 y 24,7 mN/m tras 6–24 h de cultivo respectivamente.

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1. Introduction

Lactic acid bacteria (LAB) are Gram-positive cocci or rods, which are Generally Recognized as Safe by the FDA (US Food and Drug Administration) and produce lactic acid as the major or sole fermentation product (Von Wright & Axelsson, 2012). They comprise a large number of species involved in the production of fermented milk products that belong mainly to the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus* (Mayo et al., 2010).

It is important to note that although lactic acid is the major metabolite produced by LAB, other biomolecules can simultaneously be produced depending on the species such as peptides called bacteriocins (Arauz, Jozala, Mazzola, & Penna, 2009). Bacteriocins produced by LAB are proteins or protein complexes with antimicrobial activity and therapeutic potential, even against the cancer (Moreno, Lerayer, Baldini, & Leitão, 2000; Panesar, Kennedy, Gandhi, & Bunko, 2007; Parada, Caron, Medeiros, & Soccol, 2007). The economic importance of bacteriocins in food preservation has increased their exploitation as food preservatives (Rosa & Franco, 2002).

Currently, the most widely studied and industrially exploited bacteriocin is nisin, which is produced by certain strains of the probiotic bacterium *Lactococcus lactis*, is classified as a lantibiotic, contains 34 amino acids and has a molecular weight of 3.5 kDa (Arauz et al., 2009; Oladunjoye, Singh, & Ijabadeniyi, 2016). Nisin is able to inhibit pathogens and contaminants and is the only bacteriocin internationally legalized for use as a natural biopreservative of foods, since its intake does not exert toxic effects to the human body, does not alter the ecology of the gastrointestinal tract, and, then, does not present the same risks related to the use of antibiotics (Yoneyama & Katsumata, 2006).

Several studies have reported LAB as producers not only of bacteriocins but also of biosurfactants and stressed their potential application in food processing and public health (Gudiña, Teixeira, & Rodrigues, 2011; Portilla-River, Moldes, Torrado, & Domínguez, 2008; Rodrigues, Teixeira,

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& Oliveira, 2006b; Rodrigues, Teixeira, Oliveira, & Van Der Mei, 2006a; Rodríguez, Salgado, Costés, & Domínguez, 2010; Rodríguez-Pazo, Salgado, Cotéz-Diéguez, & Domínguez, 2013; Sabo, Converti, Todorov, Domínguez, & Oliveira, 2015; Sharma, Saharan, Chauhan & Bansal, 2014). Recent studies describe the ability of different probiotic LAB to produce biosurfactants extracellularly, intracellularly, or even adsorbed on their plasma membrane (Ceresa et al., 2015; Galabova, Sotirova, Karpenko, & Karpenko, 2014; Rodrigues, Teixeira, Van De Mei, & Oliveira, 2006c; Sharma, Saharan, Chauhan, Bansal, & Procha, 2014), among which the best known is just *L. Lactis* (Machado, Mohideen, Saravanakumari, & Prabhavathi, 2013).

The commercial interest and industrial use of biosurfactants are increasing due to their advantages over chemically synthesized surfactants, including high biodegradability, low toxicity, and effectiveness even at extreme temperatures and pH (Bustos, De La Torre, Moldes, Cruz, & Domínguez, 2007; Souza, Penna, & Oliveira, 2014). Furthermore, biosurfactants also find a wider range of applications in the pharmaceutical, cosmetic, food and oil industries, as dispersant, foaming, emulsifying, micelle-forming agents, and for their anti-adhesive action against pathogens (Banat et al., 2010; Cortés-Sánchez, Hernández-Sánchez, & Jaramillo-Flores, 2013; Jhaa, Sanket, Joshia, & Geetha, 2016).

Since this bacterium is able to simultaneously produce bacteriocins and biosurfactants (Rodríguez et al., 2010), the need was seen in this study to investigate factors that may address to the production of one metabolite or the other depending on desired product.

Cell growth and metabolite accumulation are highly influenced by the culture medium composition and the operating conditions such as pH, temperature, dissolved oxygen concentration, and degree of aeration. Therefore, the influence of different parameters makes it difficult to optimize biotechnological processes (Chen, Juang, & Wei, 2015; Desai & Banat, 1997; Li, Bai, Cai, & Ouyang, 2002). In some cases, biosurfactant production can be mainly regulated by pH and growth temperature (Mulligan, Sharma, Mudhoo, & Makhijani, 2014).

LAB have optimum growth temperature between 20°C and 45°C and survive at pH 5 or less, hence demonstrating high acid tolerance, depending on the selected microorganism (Hofvendahl, Niel, & Hahn-Hägerdal, 1999). The importance of pH control in the simultaneous biosurfactant and bacteriocin production by *L. lactis* was studied by Rodríguez et al. (2010), who observed that at 30°C under pH control at 6.8 only biosurfactant was produced, while both metabolites were produced without pH control. However, these authors did not assess the effect of temperature, which can exert great influence on the production of these biomolecules, favoring the production of one or the other.

In this context, the objective of this study was to identify the effects of temperature (30°C, 37°C and 40°C) and pH control on the production of biosurfactant, bacteriocins, and lactic acid by *L. lactis* subsp. *lactis* CECT-4434, which has the ability to simultaneously synthesize these metabolites, thus contributing to the profitability of the biotechnological process under investigation.

2. Materials and methods

2.1. Microorganisms and culture conditions

Lactococcus lactis subsp. *lactis* CECT-4434 and the indicator strain *Staphylococcus aureus* CECT-239 were obtained from the Spanish Type Culture Collection (Valencia, Spain), while the other indicator strain *Lactobacillus sakei* ATCC 15521 from the American Type Culture Collection (Manassas, VA, USA). All strains were maintained under cryopreservation at −70°C in culture media specific for each microorganism, supplemented with 20% glycerol (v/v).

For *L. lactis* inoculum, 1.0 mL of stock culture was incubated at 37°C and 100 rpm in 250 mL-Erlenmeyer flasks containing Man-Rogosa-Sharpe broth (MRS, Difco, NJ, USA) composed of (g/L): 10.0 peptone, 10.0 meat extract, 5.0 yeast extract, 20.0 dextrose, 1.0 polysorbate, 2.0 ammonium citrate, 5.0 sodium acetate, 0.1 magnesium sulfate, 0.05 manganese sulfate, and 2.0 dibasic potassium phosphate.

After reaching an optical density (O.D.) of 0.8–0.9 at 600 nm, 12 mL of this cell suspension were inoculated into 500 mL Erlenmeyer flasks containing 120 mL of MRS broth. The flasks were incubated under stirring at 100 rpm for 24 h at 30°C, 37°C or 40°C, with or without pH control at 5.0–5.3 using 1.0% $CaCO_3$, and samples of culture medium were collected at fixed times (6, 16, and 24 h). The experiment was performed in triplicate.

The pre-inoculum of *L. sakei* and *S. aureus* was carried out in MRS broth (Difco) and Tryptic Soy Broth (TSB, Difco), respectively, under the same conditions described above for *L. lactis*, up to the achievement of O.D. of 0.3–0.4 at 600 nm.

2.2. Bacteriocin extraction

After centrifugation (15.000 *g* for 15 min at 4°C) of fermented broth samples and biomass separation, the supernatant had the pH adjusted to 6.0–6.5, was filtered with membrane filter with 0.45 µm-pore diameter (Millipore, Billerica, MA, USA), transferred to Eppendorf tubes, subjected to heating for 3 min at 80°C to inhibit proteases, and stored at 4°C for later analysis.

The antimicrobial activity of produced bacteriocin was analyzed by the diffusion technique in wells. To 150 µL of either indicator strain, *L. sakei* ATCC 15521 or *S. aureus* CECT-239, were inoculated in MRS and TSB, respectively, both containing 1% agar. Wells of 7 mm in diameter were cut in the gelled medium with the aid of a sterile metal cylinder and filled with 50 µL of each extract obtained as previously described. After the plates had been incubated for 24 h at 37°C, the inhibition zones evidenced by bright areas around the wells (halos) were measured with the aid of a caliper.

2.3. Extraction and analysis of biosurfactant

After centrifugation at 4470 *g* for 10 min at 10°C, the supernatant was recovered for measurement of extracellular biosurfactant. To extract intracellular biosurfactant and/or the adhered one, cells were washed with deionized water, resuspended in phosphate saline buffer PBS (10 mM KH_2PO_4/K_2HPO_4 and 150 mM NaCl, pH 7.0) at a ratio of 1.0 mL of PBS per 6.0 mL of the initial sample, and subjected to stirring for 2 h at 25°C. After that time, samples were centrifuged at 4470 *g* for

10 min and 25°C, and the supernatants surface tension (ST) was measured.

ST either of cell-free culture medium or extract in PBS was measured by the ring method, using a tensiometer, model Krüss K9 (Krüss, Hamburg, Germany), equipped with platinum *de Noüy* ring with 1.9 cm diameter. ST values represent the average of three independent measurements at 25°C.

2.4. Determinations of glucose and lactic acid

After filtration of samples through membrane filters (Millipore) with 0.45 µM-pore diameter, glucose and lactic acid concentrations were determined at 30°C by a high performance liquid chromatography (HPLC), model LC-20A Prominence (Shimadzu, Kyoto, Japan), equipped with a separation column, model Supelcogel C-610H (Supelco, Bellefonte, PA, USA) (H 30 cm x 7.8 mm, 9 µM particle), using as eluent an aqueous solution of 1% H_3PO_4 at a flow rate of 0.5 mL/min. Glucose was quantified using standard curves obtained beforehand with high purity standard solutions for use in HPLC.

3. Results and discussion

Various external parameters can interfere with the production and effective throughput of both biosurfactants as bacteriocins, including culture medium composition, temperature, pH, and incubation time (Cortés-Sánchez et al., 2013).

In this context, a series of batch fermentations was performed in MRS broth in order to identify the effects of temperature (30°C, 37°C, and 40°C) and pH control on the productions of biosurfactant, bacteriocin, and lactic acid by *Lc. lactis* CECT-4434, to determine the best conditions for producing each of these metabolites.

3.1. Lactic acid production

Table 1 shows the pH values obtained as a function of time in runs carried out with or without pH control in the range 5.0–5.3 at different temperatures. It can be seen that, in the absence of control, the pH values at 30°C were close to those obtained at 37°C, while at 40°C there was a smaller reduction in pH over time, probably due to the scarce of lactic acid production (under 15 g/L in all cases), as it can be seen in Figure 1, resulting from a metabolism slowdown at this relatively high temperature. On the other hand, the pH control favored the uptake of glucose as carbon source, allowing an increase of lactic acid concentration of 38.8%

Table 1. pH changes as a function of time during cultures carried out at 30°C, 37°C, and 40°C, with or without pH control.

Tabla 1. Variación del pH a lo largo del tiempo durante el cultivo de *Lc. Lactis* llevado a cabo a 30, 37 y 40°C, con y sin control de pH.

	Time (h)	30°C	37°C	40°C
Without pH control	0	5.30	5.30	5.30
	6	4.74	4.71	4.99
	16	4.23	4.25	4.77
	24	4.16	4.20	4.64
With pH control	0	5.30	5.30	5.30
	6	5.11	5.05	5.12
	16	5.10	5.01	5.15
	24	5.21	5.12	5.14

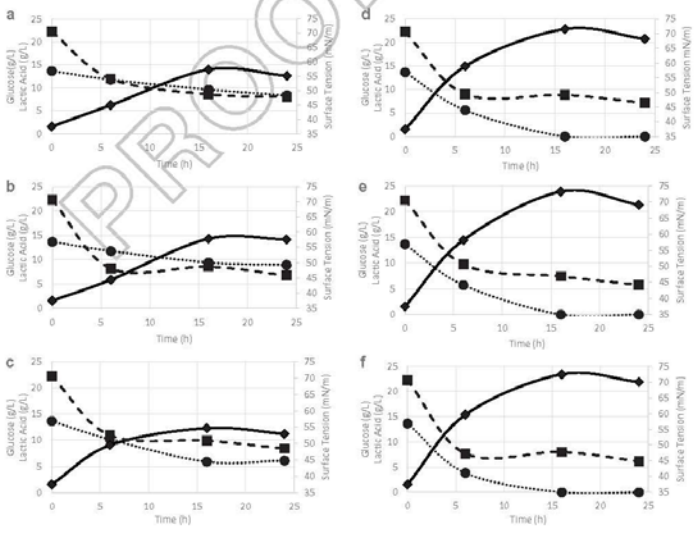

Figure 1. Glucose consumption (•), surface tension reduction (□), lactic acid production (♦). Runs without pH control at: (a) 30°C, (b) 37°C, and (c) 40°C. Runs with pH control at: (d) 30°C, (e) 37°C, and (f) 40°C.

Figura 1. Consumo de glucosa (•), reducción de tensión superficial (□), producción de ácido láctico (♦). Ensayos sin control del pH: (a) 30°C, (b) 37°C and (c) 40°C. Ensayos con control del pH: (d) 30°C, (e) 37°C y (f) 40°C.

at 30°C, 40.3% at 37°C, and 47.4% at 40°C compared with runs performed without control, with a maximum value (23.4 g/L) after 16 h of cultivation at 40°C. As expected by the homofermentative metabolism of *L. lactis*, lactic acid production was proportional to glucose consumption.

The same effect was observed by Rodríguez et al. (2010) for the same strain, which produced, in bioreactor under pH control (6.8), 23 g/L of lactic acid in 6 h at 30°C, with complete glucose consumption, while the absence of control reduced the consumption and delayed lactic acid production (7.8 g/L in 30 h).

3.2. Bacteriocin production

Penna, Jozala, Gentille, Pessoa, and Cholewa (2006) observed a strong influence of pH of the culture medium on nisin release by *L. lactis* ATCC 11454, that is, at pH <6, more than 80% of it was released extracellularly, while at pH >6, it was mainly linked to cell membrane or intracellularly trapped. According to these results, nisin production by *L. lactis* subsp. *lactis* IT-4 achieved a maximum at pH 5 and decreased at higher pH (Sadiq et al., 2014), which confirms the validity of investigating, in the present study, the effect of pH control in the range 5.0–5.3.

Table 2 shows that there was no statistically significant difference between the values of extracellular activity of bacteriocin produced by *L. lactis* CECT-4434 in cultured performed at 30°C and 37°C, with or without pH control, expressed as inhibition diameter of growth of *Lactobacillus sakei* ATCC 15521 (9.0–10.6 mm) and *Staphylococcus aureus* CECT-239 (12.5–14.0 mm). The lowest activity was observed at 40°C, especially without pH control, resulting in inhibition halos of only 7.5 mm against *L. sakei* and 11.0 mm against *S. aureus* after 24 h of cultivation. It is also noteworthy that the largest inhibition diameters were obtained with broth samples mainly taken after 6 h, that is, during the exponential growth phase (Table 3), while the narrowest one with stationary phase samples, suggesting possible bacteriocin degradation by the microorganism under conditions of substrate starvation.

These results agree qualitatively with those of Sadiq et al. (2014), who observed at 25–30°C maximum antimicrobial activity of *L. lactis* PI-2 (8.0–10.0 mm) nisin against *L. lactis* IT-4 and a decrease to approximately 6.0 mm at 33–37°C. Comparison with such a thermal profile suggests a certain thermostability of bacteriocin produced in this study.

3.3. Biosurfactant production

In addition to bacteriocins, LAB may produce biosurfactants extracellularly, intracellularly, or adsorbed on their plasma membrane. Although there are many studies describing the ability of different LAB to produce biosurfactants, the most important biosurfactant producer is *L. Lactis* (Rodrigues et al., 2006a).

In particular, Rodrigues et al. (2006a) reported the production of extracellular biosurfactant by *L. lactis* 53, while Rodríguez et al. (2010) reported no extracellular biosurfactant

Table 2. Inhibition zones (mm) of *Lactobacillus sakei* ATCC 15521 and *Staphylococcus aureus* CECT-239 growth induced by *Lactococcus lactis* CECT-4434 bacteriocin produced at different temperatures, with and without pH control.

Tabla 2. Zonas de inhibición de crecimiento (mm) de las cepas *Lactobacillus sakei* ATCC 15,521 y *Staphylococcus aureus* CECT-239 por la bacteriocina producida por *Lactococcus lactis* CECT-4434 a diferentes temperaturas, con y sin control de pH.

		L. sakei ATCC 15521			*S. aureus* CECT-239		
	Time (h)	30°C	37°C	40°C	30°C	37°C	40°C
Without pH control	6	10.0 ± 0.0^{def}	9.0 ± 0.0^{bc}	7.8 ± 0.8^{ab}	14.0 ± 0.0^{l}	13.4 ± 0.4^{jkl}	12.9 ± 0.4^{ijkl}
	16	9.8 ± 0.3^{cdef}	9.6 ± 0.2^{cde}	7.7 ± 0.9^{ab}	13.0 ± 0.0^{hijkl}	12.5 ± 0.1^{hi}	12.0 ± 0.0^{gh}
	24	9.8 ± 0.3^{cdef}	9.4 ± 0.1^{cd}	7.5 ± 0.6^{a}	13.8 ± 0.2^{kl}	12.8 ± 0.3^{hijk}	11.0 ± 0.0^{fg}
With pH control	6	10.5 ± 0.0^{ef}	10.0 ± 0.0^{cdef}	9.6 ± 0.2^{cde}	14.0 ± 0.0^{l}	13.1 ± 0.2^{ijkl}	12.7 ± 0.0^{hij}
	16	10.6 ± 0.1^{ef}	10.4 ± 0.0^{def}	10.0 ± 0.1^{def}	13.6 ± 0.6^{kl}	13.0 ± 0.0^{hijkl}	12.4 ± 0.7^{hij}
	24	10.4 ± 0.5^{ef}	10.2 ± 0.0^{def}	10.0 ± 0.0^{cdef}	14.0 ± 0.0^{l}	12.8 ± 0.7^{ijkl}	13.2 ± 0.1^{ijkl}

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*Mean values ($n = 3$) ± standard deviation. Different letters show statistically significant difference among the values in the table ($P < 0.05$).

*Valores medios (n = 3) ± desviación estándar. Diferentes letras muestran diferencias estadísticamente significativas entre los valores de la tabla (P < 0,05).

Table 3. *Lactococcus lactis* CECT-4434 biomass concentration (g/L) and reduction of surface tension (STred) as a result of biosurfactant production at different temperatures, with and without pH control.

Tabla 3. Concentración de biomasa de *Lactococcus lactis* CECT-4434 (g L) y reducción de la tensión superficial (STred) como resultado de la producción de biosurfactante a diferentes temperaturas, con y sin control del pH.

		30°C 37°C		40°C			
	Time (h)	Biomass (g/L)	STred	Biomass (g/L)	STred	Biomass (g/L)	STred
Without pH control	0	0.1 ± 0.0^{a}	–	0.1 ± 0.0^{a}	–	0.1 ± 0.0^{a}	–
	6	4.6 ± 0.1^{f}	16.4 ± 0.5^{a}	4.8 ± 0.0^{f}	22.5 ± 0.5^{cdef}	2.3 ± 0.1^{b}	18.1 ± 0.5^{ab}
	16	5.3 ± 0.1^{g}	21.9 ± 0.2^{cde}	5.4 ± 0.2^{gh}	24.4 ± 2.5^{fgh}	3.3 ± 0.0^{c}	19.7 ± 1.7^{bc}
	24	5.7 ± 0.2^{hi}	22.8 ± 1.0^{cdef}	5.4 ± 0.0^{g}	24.7 ± 2.0^{fgh}	3.6 ± 0.1^{d}	22.1 ± 0.7^{cde}
With pH control	0	0.1 ± 0.0^{a}	–	0.1 ± 0.0^{a}	–	0.1 ± 0.0^{a}	–
	6	5.4 ± 0.0^{g}	21.1 ± 1.2^{cd}	6.0 ± 0.1^{i}	20.0 ± 0.5^{bc}	4.6 ± 0.1^{f}	23.3 ± 1.0^{defg}
	16	6.8 ± 0.0^{k}	21.4 ± 3.5^{defgh}	7.1 ± 0.1^{l}	23.7 ± 1.4^{defgh}	4.0 ± 0.1^{e}	22.8 ± 0.9^{cdef}
	24	6.5 ± 0.1^{j}	24.2 ± 1.3^{efgh}	6.4 ± 0.1^{j}	26.4 ± 1.6^{h}	3.9 ± 0.1^{e}	25.7 ± 1.2^{gh}

*Mean values ($n = 3$) ± standard deviation. Different letters show statistically significant difference among the values in the table ($P < 0.05$). Intracellular biosurfactant and/or biosurfactant adsorbed onto cell membrane was extracted with PBS (pH 7.0).

*Valores medios (n = 3) ± desviación estándar. Diferentes letras muestran diferencias estadísticamente significativas entre los valores de la tabla (P < 0,05). El biosurfactante intracelular y/o adsorbido sobre la membrana celular se extrajo con PBS (pH 7,0).

production by *L. lactis* CECT-4434, even though observed the production of biosurfactant adsorbed onto the cells after extraction with PBS (pH 7).

Based on these results, we investigated in this study the biosurfactant production by the latter strain measured as capacity of reducing the culture medium surface tension (STred), either with or without pH control, but the ST variations (not shown) were not statistically significant, thus confirming the observations of Rodríguez et al. (2010).

On the other hand, runs performed either with or without pH control showed interesting results in terms of production of intracellular biosurfactant and/or biosurfactant adsorbed onto the cell membrane after extraction with PBS (pH 7.0) (Table 3).

Biosurfactant production was associated to cell growth in the present study. Despite of the slow growth of *L. lactis* CECT-4434 at 40°C (0.1–4.6 g/L), biosurfactant production was satisfactory in the culture performed with pH control (Table 3), being STred after 6 h of cultivation (23.3 mN/m) appreciably higher than without control (19.1 mN/m). Indeed, it is worth remembering that a substance can be considered a biosurfactant if is able to reduce the ST by no less than 8 mN/m (Van Der Vegt, Van Der Mei, Noordmans, & Busscher, 1991).

The pH control allowed for better cell growth at all tested temperatures (21.5% higher as an average), and the highest cell concentration (7.1 ± 0.1 g/L) was obtained at 37°C after 16 h. Possibly, without any pH control, the acidity of the medium may have inhibited the growth of microorganism (Parada et al., 2007).

The culture at 30°C with pH control showed higher STred after only 6 h (21.1 mN/m) compared with the one without pH control, which took more than twice as long to achieve the same reduction at the same temperature. On the other hand, cultivations at 37°C showed the opposite trend. In particular, with pH control STred after 6 h of culture (20.0 mN/m) was 11% lower and cell growth (6.0 g/L) 20% higher than without pH control.

In agreement with these results, Rodrigues et al. (2006a) observed that biosurfactant production by *L. lactis* 53 was associated with growth. However, Rodríguez et al. (2010) reported that, although ST decreased since the beginning of fermentation as a result of metabolism and growth, it achieved its maximum value (31.1 mN/m) at pH 6.8 in the early stationary phase, but then decreased to only 17.5 mN/m due to possible consumption of biosurfactant as a carbon source under conditions of substrate starvation. No similar decrease was observed in this study during the stationary phase of growth.

4. Conclusions

In this work, we studied the behavior of *L. lactis* in cultures performed at different temperatures with or without pH control. Under both conditions, the temperature range 30–37°C allowed for the largest bacteriocin production, as evidenced by the formation of inhibition zones against *Lactobacillus sakei* and *Staphylococcus aureus* 5–22% and 4–14% higher, respectively, compared with 40°C. At temperature of 37°C, without pH control, would be the best choice for mainly favoring biosurfactant production, as evidenced by a reduction in ST between 22.5 and 24.7 mN/m after 6–24 h of culture. In addition, this temperature would allow producing bacteriocin with satisfactory antimicrobial activity (mean inhibition zone of 9.3 and 12.9 mm against *L. sakei* and *S. aureus*, respectively) and lactic acid at concentration of 14.28 g/L, thus contributing to the economy of the process.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Production of biosurfactants from vine-trimming shoots using the halotolerant strain *Bacillus tequilensis* ZSB10

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ABSTRACT

The strain *Bacillus tequilensis* ZSB10, isolated from Mexican brines, was able to grow and produce extracellular and cell-bound biosurfactants using nine culture broths formulated from hydrolyzates obtained from the cellulosic and hemicellulosic fractions of vine-trimming wastes. The results confirm its halotolerance since it managed to grow both in the presence and absence of salts. It also was able to consume sugars such as glucose and xylose. The process was then scaled up into a 2-L bioreactor using the mixture of hemicellulosic (50%) and cellulosic hydrolyzate (50%) supplemented with mineral salt medium as culture medium at different biomass concentrations. Crude extracellular biosurfactant yielded 1.52 g/L and lowered the surface tension to 38.6 mN/m with a critical micelle concentration of 177.14 mg/L. Furthermore, it was able to emulsify with kerosene after 24 h (E_{24} = 47%). Crude cell-bound biosurfactant only yielded 0.0783 g/L and showed lower emulsifying characteristics than extracellular biosurfactant (E_{24} = 41% with kerosene).

1. Introduction

Biosurfactants are amphiphilic molecules produced by microorganisms that tend to reduce surface and interfacial tension of solutions and to form emulsions (Shavandi et al., 2011; Freitas et al., 2009). Bacteria, fungi and yeasts can produce biosurfactants during growth on a variety of substrates, leading to products with various biochemical and structural characteristics that can remain attached to the cell surface of the microorganism (cell-bound biosurfactants), or be expelled into the culture medium (extracellular biosurfactants) (Shavandi et al., 2011; Mukherjee et al., 2006). The surfactant-producing microorganism, culture medium (carbon source, nitrogen, phosphorus, trace elements such as Mg, Fe or Mn) and growth conditions (such as temperature, aeration or pH) determine the amount and composition of the biosurfactant (Bodour and Maier, 2003).

Biosurfactants have advantages over their synthetic counterparts since they are biodegradable, do not accumulate in the environment, have lower toxicity and, most importantly, these exhibit higher physical and chemical properties (emulsifying capacity, tolerance to pH, temperature and ionic strength) compared to those of the synthetic surfactants. However, it is necessary to find an efficient production process in order to make biosurfactants economically competitive with lower-cost chemical surfactants (Nitschke and Costa 2007; Satpute et al., 2010; Franzetti et al., 2011). Because they contain high levels of carbohydrates or lipids that favor bacterial growth and biosurfactant synthesis, agro-

Abbreviations: Abs_{600nm}, absorbance 600 nm; BS, biosurfactant; CMC, critical micelle concentration; E_{24}, emulsification index (24 h in repose); FPU, filter paper units; HMF, hydroxymethylfurfural; HPLC, high-performance liquid chromatography; MSM, mineral salt medium; MSM + GLU, mineral salt medium plus 15 g/L of glucose; MSM + XYL, mineral salt medium plus 15 g/L of xylose; MSM + HH, mineral salt medium plus hemicellulosic hydrolyzate; HH, hemicellulosic hydrolyzate; MSM + CH, mineral salt medium plus cellulosic hydrolyzate; CH, cellulosic hydrolyzate; MSM + HH + CH, mineral salt medium plus mixture of hydrolyzates (50% hemicellulosic hydrolyzate and 50% cellulosic hydrolyzate); HH + CH, mixture of hydrolyzates (50% henicellulosic hydrolyzate and 50% cellulosic hydrolyzate); ST, surface tension; ΔST, surface tension reduction.

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industrial byproducts can be low-cost, sustainable alternatives for the production of biosurfactants (Makkar and Cameotra, 2002).

The wine industry produces approximately 1.7 t/ha/year of vine-trimming shoots in Spain, and new processes have been developed to decrease the environmental impact of those residues. According to Max et al. (2010) vine-trimming shoots had the following composition: cellulose (34.0%), hemicelluloses (19.0%), lignin (27.1%), extracts (7.1%) and other minor compounds (12.7%). The valorization of vine-trimming shoots through production of a great variety of products, including biosurfactants, has been carried out in several studies (Devesa-Rey et al., 2011). Hydrolysis of these residues and further fermentation of hemicellulosic sugars by *Lactobacillus* and *Debaryomyces* strains to produce lipopeptides as biosurfactants has been carried out by several authors (Moldes et al., 2007; Bustos et al., 2007; Portilla-Rivera et al., 2008). However, low yields of cell-bound biosurfactants were obtained and additional treatments had to be done for their detachment (Vecino et al., 2015).

Bacillus strains have been shown to produce higher yield of lipopeptide as biosurfactant than those obtained with *Lactobacillus* strains using several agro-industrial wastes (Lima de Franca et al., 2015); but performance of *Bacillus* in fermentation process using the vine-trimming shoots waste as substrate is unknown.

In order to provide additional alternatives for valorization of vine-trimming shoots, the *Bacillus tequilensis* ZSB10 strain, recently isolated from brines at Zapotitlán Salinas (Puebla), Mexico, was investigated regarding its ability to use vine-trimming shoots as carbon source for the production of lipopeptides as biosurfactants. Halotolerant bacteria, such as *B. tequilensis*, have physiological properties that facilitate commercial exploitation: limited nutritional requirements, a variety of chemical energy sources, and resistance to contamination (Ramírez et al., 2004).

2. Materials and methods

2.1. Microorganism and culture conditions

B. tequilensis ZSB10 was obtained from ENCB-IPN WDCM449 culture collection (ENCB-RP-001). The strain was isolated from brines in Zapotitlán Salinas, Puebla, Mexico. It was reactivated according to the procedure reported by Saini et al. (2008), using mineral salt medium (MSM) containing (g/L): $NaNO_3$ (2.97), $MgSO_4{\cdot}7H_2O$ (0.4), KCl (1.0), $CaCl_2{\cdot}2H_2O$ (0.1), Na_2HPO_4 (24.3), NaCl (20) and yeast extract (10). The medium was adjusted to pH 7.4 and autoclaved at 121 °C for 15 min. 250 mL Erlenmeyer flasks were charged with 50 mL of culture medium and were incubated under agitation of 150 rpm in a constant temperature incubator shaker (Optic Ivymen System, Comecta S.A., distributed by Scharlab, Madrid, Spain) at 35 °C for 48 h.

2.2. Raw material

Samples of vine-trimming shoots were dried at room temperature, milled to a particle size less than 1 mm, homogenized in a single batch to avoid compositional differences and stored until use by sequential treatments in order to take advantage of both the cellulosic and the hemicellulosic fractions.

2.2.1. Pre-hydrolysis

The first stage consisted of a treatment with dilute acid to solubilize hemicelluloses. The raw material underwent autoclaving (Sterilclav-70 Trade Raypa, Barcelona, Spain) under the following conditions: sulfuric acid 2%, 130 °C for 15 min using a liquid/solid ratio 8 g/g, according to optimized conditions (Bustos et al., 2004). The product was filtered and the liquid phase was brought up to pH 6.0 using calcium carbonate, filtered again, and finally the liquid phase was treated with activated charcoal (10 g of hydrolyzate/1 g of activated carbon), stirred at 150 rpm, 1 h at 25 °C, and finally filtered to obtain clear liquors. The supernatant, composed mainly of xylose and glucose and smaller amounts of inhibitors such as acetic acid, furfural and HMF (hydroxymethylfurfural), was used as a culture medium.

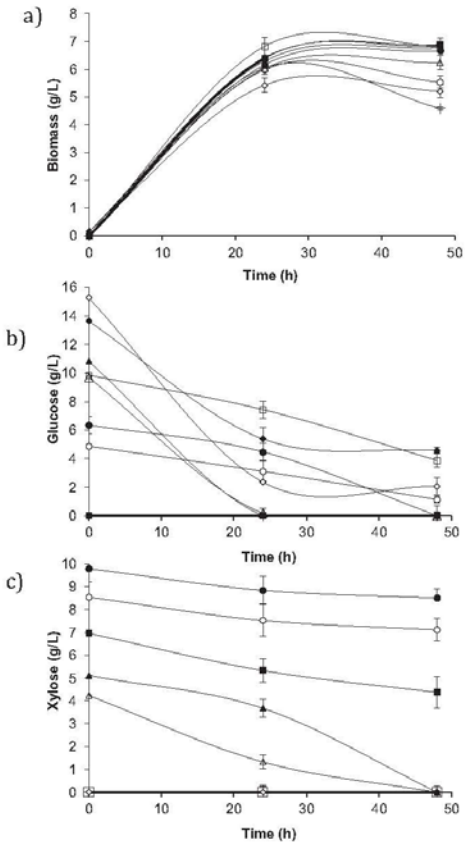

Fig. 1. Kinetics of ZSB10 strain in different culture media under the following culture conditions: 35 °C, 150 rpm during 48 h. (a) Bacterial growth; (b) glucose consumption; and (c) xylose consumption. MSM (♦); MSM + GLU (□); MSM + XYL (■); MSM + HH (○); HH (●); MSM + CH (◊); CH (♦); MSM + HH + CH (△); HH + CH (▲).

2.2.2. Alkaline stage

Solids from pre-hydrolysis, containing the cellulosic fraction and lignin, were delignified to increase enzymatic digestibility. The solid was washed with distilled water to adjust to pH 6.0, oven-dried at 50 °C for 72 h, and treated in autoclave with solutions containing 8% NaOH at 130 °C during 120 min according to the conditions reported by Bustos et al. (2005). In this step, the liquor/solid ratio was fixed in 10 g/g. At the end of treatments, the solid residue containing the cellulose fraction was separated by filtration, washed with water, air dried at room temperature and used for enzymatic hydrolysis.

Table 1
Initial composition of culture broths used for the fermentation of strain ZSB10 (g/100 mL).

	MSM	MSM + GLU	MSM + XYL	MSM + HH	HH	MSM + CH	CH	MSM + HH + CH	HH + CH
$NaNO_3$ (g)	0.30	0.30	0.30	0.30		0.30		0.15	
$MgSO_4 \cdot 7H_2O$ (g)	0.04	0.04	0.04	0.04		0.04		0.02	
KCl (g)	0.10	0.10	0.10	0.10		0.10		0.05	
$CaCl_2 \cdot 2H_2O$ (g)	0.01	0.01	0.01	0.01		0.01		0.005	
Na_2HPO_4 (g)	2.43	2.43	2.43	2.43		2.43		1.22	
Yeast extract (g)	1.00	1.00	1.00	1.00		1.00		0.5	
NaCl (g)	2.00	2.00	2.00	2.00		2.00		1.00	
Glucose (g)		1.5							
Xylose (g)			1.5						
Hemicellulosic hydrolyzate (HH), (mL)				94.13	100			47.06	50
Cellulosic hydryolyzate (CH), (mL)						94.13	100	50	50
Water (mL)	94.13	92.63	92.63						

MSM: Mineral salt medium.
MSM + GLU: Mineral salt medium plus 15 g/L of glucose.
MSM + XYL: Mineral salt medium plus 15 g/L of xylose.
MSM + HH: Mineral salt medium plus hemicellulosic hydrolyzate.
HH: Hemicellulosic hydrolyzate.
MSM + CH: Mineral salt medium plus cellulosic hydrolyzate.
CH: Cellulosic hydrolyzate.
MSM + HH + CH: Mineral salt medium plus mixture of hydrolyzates (50% hemicellulosic hydrolyzate and 50% cellulosic hydrolyzate).
HH + CH: Mixture of hydrolyzates (50% hemicellulosic hydrolyzate and 50% cellulosic hydrolyzate).

2.2.3. Enzymatic hydrolysis

Two commercial enzymatic concentrates ("Celluclast" and "Novozyme 188", with cellulase and β-glucosidase acitivities, respectively), kindly provided by Novozymes, Denmark, were used to hydrolyze the cellulosic fraction in order to obtain glucose solutions. The cellulase activity of concentrates was measured by the Filter Paper Activity test according to Mandels et al. (1976) and expressed as Filter Paper Units (FPU)/mL, while the β-glucosidase activity was measured according to Paquot and Thonart, (1982) and was expressed as International Units per milliliter (IU/mL). The operational conditions used in the enzymatic hydrolysis were: temperature 48.5 °C, pH 4.85 (adjusted with citrate buffer), liquor/solid ratio 30 g/g, cellulase-substrate ratio 28 FPU/g and cellobiase/cellulase ratio 13 IU/FPU at 150 rpm (Bustos et al., 2005). The hydrolysis of the cellulosic fraction, expressed as the concentration of glucose (g/L), was monitored by HPLC for 50 h. At the end of the experiment a glucose concentration of 21.57 g/L was achieved.

2.3. Biosurfactant production by the strain ZSB10 using different culture media

Table 1 summarizes the composition of the nine culture media assayed to evaluate the production of biosurfactants using the strain ZSB10. All culture media were standardized to initial pH 7.4 using 1 N NaOH solution. Then, 50 mL was placed into 250 mL Erlenmeyer flasks. All fermentation media were sterilized in autoclave (Trade Raypa SL, Terrassa, Barcelona, Spain) at 100 °C for 60 min, and further inoculated with the strain ZSB10 in a percentage of 10% of the final volume of culture using washed cells (adjusted to 3.33 g/L), to obtain an initial concentration of 0.33 g/L. All media were incubated at 35 °C, 150 rpm and sampled at specific fermentation times (24 h and 48 h), and finally centrifuged (Ortoalresa, Consul 21, EBA 20, Hettich Zentrifugen, Germany) at 2755 × g for 15 min and 4 °C, for analysis.

2.4. Scale up of biosurfactant production in a 2-L bioreactor

One liter of culture medium MSM + HH + CH (mineral salt medium with the mixture of hemicellulosic (50%) and cellulosic (50%) hydrolyzates) was prepared in order to use both the hemicellulosic and cellulosic fractions. The fermentation broth was placed in a 2-L bioreactor (Biostat B fermenter Plus, Sartorius, Germany), and inoculated with 10% of biomass (adjusted to 3.33 g/L), to give an initial biomass concentration of 0.33 g/L in the culture medium at time 0. During incubation at 35 °C and pH 7.4, the agitation speed was 150 rpm and the air flow rate was 2 L/min. Under these conditions the percentage of dissolved oxygen in medium was 0 during fermentation.

In a separate experiment to determine whether biomass concentration affects the production and activity of biosurfactant, the initial biomass concentration was increased tenfold. One liter of culture medium (MSM + HH + CH) was inoculated with 10% of biomass adjusted to 33.3 g/L, in order to start with an initial biomass concentration of 3.33 g/L in the culture medium at time 0.

2.5. Analytical methods

2.5.1. Sugar consumption analysis by HPLC

Samples (1 mL) were taken at selected fermentation times, centrifuged at 2755 × g for 15 min and 10 °C (Ortoalresa, Consul 21, EBA 20, Hettich Zentrifugen, Germany) and filter-sterilized using 0.22 μm pore-size membranes (EMD Millipore Corporation, Billerica, MA, USA). The liquid phase of the samples was employed for glucose, xylose, lactic acid, acetic acid, furfural and HMF analysis using a high-performance liquid chromatography (HPLC) system (Agilent, model 1200, Palo Alto, CA, USA) equipped with a refractive index detector and an Aminex HPX-87H ion exclusion column (Bio Rad 300 × 7.8 mm, 9 μm particles) with a guard column, eluted with 0.003 M sulfuric acid at a flow rate of 0.6 mL/min at 50 °C for 25 min.

2.5.2. Bacterial growth

Bacterial growth was monitored by measuring the turbidity of the cultures at 600 nm ($Abs_{600\,nm}$). Biomass was expressed as dry weight (g/L). In order to prepare a calibration curve, the strain ZSB10 was grown in 250 mL Erlenmeyer flasks containing 50 mL of MSM culture broth (pH 7.4) and was incubated under agitation of 150 rpm in a constant temperature incubator shaker (Optic Ivymen System, Comecta S.A., distributed by Scharlab, Madrid, Spain) at 35 °C for 48 h. The medium was centrifuged at 2755 × g, 10 °C for 15 min under sterile conditions and the precipitate was washed twice with a 4.9% NaCl solution. The absorbance of the biomass slurry was adjusted to 1.0 at a wavelength of 600 nm ($Abs_{600\,nm} = 1$) using a UV–vis Cintra 6 Spectrophotometer (GBC Scientific Equipment Ltd., Braeside, Australia). Bacterial growth was determined by triplicate measuring the absorbance of different

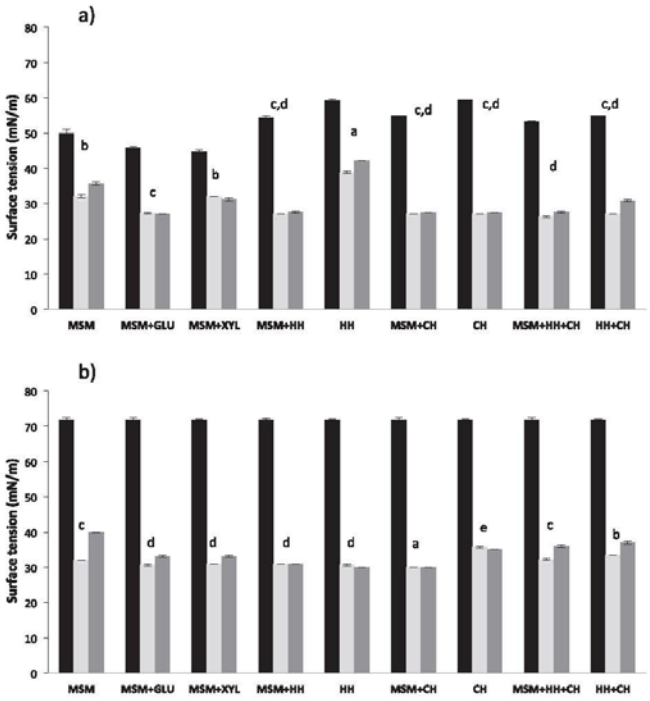

Fig. 2. Surface tension (expressed in mN/m) in (a) nine different culture media due to the production of extracellular biosurfactant, and (b) water due to cell-bound biosurfactant produced in nine different culture broths, using the ZSB10 strain. Same letters show no significant difference ($p > 0.05$). Time 0 (black); after 24 h (light grey); after 48 h (dark grey).

dilutions of the fermented culture medium at 600 nm and oven-dried at 105 °C (Celsius 2007, Memmert, Schwabach, Germany) to constant weight. The following equation was obtained: biomass dry weight (g/L) = 3.3089 (Abs_{600nm}) − 0.0578; r^2 = 0.9958.

2.5.3. Determination of surface tension of extracellular and cell-bound biosurfactants

Extracellular biosurfactant was obtained from the supernatant of the exhausted fermentation culture broth after centrifugation (Ortoalresa, Consul 21, EBA 20, Hettich Zentrifugen, Germany) at 2755 × *g* for 15 min and 4 °C. The precipitate (biomass pellet) was resuspended with distilled water in the original volume of culture broth in order to avoid dilution, washed twice with distilled water using a vortex (Classic, Velp Scientific, Italy) at 3000 rpm for 5 min, then it was centrifuged at the same conditions mentioned above to obtain cell-bound biosurfactant suspended in supernatant.

Both supernatants, extracellular and cell-bound biosurfactants, were used to determine the surface tension (ST) using a Krüss tensiometer equipped with a 1.9 cm Du Noüy wettable platinum ring at room temperature following the ring method (Kim et al., 2000). Biosurfactant activity of strain ZSB10 was determined measuring the surface tension reduction (ΔST).

2.5.4. Extraction of crude biosurfactant

The extraction of crude biosurfactant was performed using extracellular and cell-bound biosurfactant supernatants of the fermentation obtained from ZSB10 strain in the culture broth MSM + HH + CH (mineral salt medium with the mixture of hemicellulosic (50%) and cellulosic (50%) hydrolyzates) in a 2-L bioreactor for 52 h, starting with an initial biomass concentration of 0.33 g/L. Both supernatants were acidified to pH 2.0 with 6N HCl and left to stand at 4 °C overnight, afterwards these were centrifuged at 2755 × *g* for 15 min at 4 °C; the precipitates, extracellular and cell-bound biosurfactant were redissolved in distilled water (60 mL) and added to a separating funnel with dichloromethane (60 mL), that is, in a ratio of distilled water:dichloromethane (1:1). The organic phase was evaporated in order to obtain both crude biosurfactants as powders (Najafi et al., 2010; Shavandi et al., 2011).

2.5.5. Determination of critical micelle concentration (CMC) and emulsification index E_{24} in crude biosurfactants

Extracellular and cell-bound biosurfactants as crude powders were used to determine the CMC and emulsification index E_{24} using kerosene as hydrocarbon. The re-suspended biosurfactant powders were slightly soluble in water, in order to increase their solubility, the pH of the biosurfactant solutions should be increased to 10.5 using a solution of 3 M NaOH before CMC and E_{24} analysis. For CMC analysis, crude biosurfactant solutions at 180, 160, 140, 120, 100, 80, 60, 40, 20 and 10 ppm were prepared and the ST at each concentration was measured. Then, the ST values were plotted and the CMC was calculated by the intersection of the lines formed by the points of measurement.

The emulsification index (E_{24}) was determined in duplicate by mixing 1 mL of kerosene and 1 mL of crude biosurfactant solution

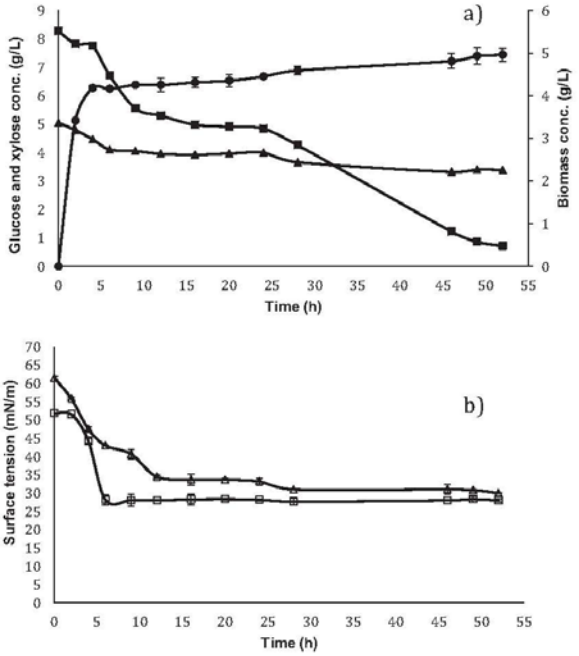

Fig. 3. Biosurfactants production by the ZSB10 strain using the culture medium MSM + HH + CH (mineral salt medium with the mixture of hemicellulosic (50%) and cellulosic (50%) hydrolyzates) in a 2-L bioreactor starting with an initial biomass concentration of 0.33 g/L. Incubation conditions: 35 °C, 150 rpm, and pH 7.4. Glucose consumption (■); xylose consumption (▲); biomass (●); surface tension of extracellular biosurfactant (□); surface tension of cell-bound biosurfactant (△).

(1 mg/mL) in a 15 mL Falcon screw-capped tube (17 × 120 mm). Tubes were stirred for two minutes using a vortex to create emulsion and left to stand stable for 24 h. Then, the E_{24} was calculated by dividing the height of the emulsion layer by the height of the total mixture, and multiplied by 100 (Techaoei et al., 2011).

3. Results and discussion

3.1. Production of biosurfactants by ZSB10 strain using different growth media

Fig. 1a shows the growth of bacterial strain ZSB10. This growth appeared significant during the first 24 h (exponential growth phase), being able to grow in the 9 different culture media assayed, although the behavior was different depending on the medium composition. The strain was favored when the mineral salt medium (MSM) was added to a carbon source such as glucose or xylose, but was also able to grow well without the salts and yeast extract contained in MSM medium, meaning that could grow using only the cellulosic or hemicellulosic sugars provided by hydrolyzates from vine-trimming shoots. The results demonstrate the halotolerance of this strain, which means that it could grow either in the absence or in presence of salts. Furthermore, biosurfactants production was evaluated using the extracellular biosurfactant excreted into the culture medium and the cell-bound biosurfactants by washing the cells with distilled water. Fig. 2a shows that at time 0 (ST_0) there was a reduction of the ST regarding the value of the water (72 mN/m), which must be attributed to the components of the culture medium. A significant reduction in ST was observed on day 1 due to the concentration of excreted biosurfactant to the medium, meanwhile on day 2 a visible increase in ST was observed in most cases, which may be due to the microorganism starts consuming the metabolite. At each time surface tension reduction (△ST) was calculated as ST_0–ST_t. The △ST of CH culture medium showed the highest value (△ST = 59.5–27 = 32.5 mN/m), followed by MSM + CH and HH + CH (△ST = 55–27 = 28 mN/m) and MSM + HH (△ST = 54.3–27 = 27.3 mN/m). In all cases, ST was 27 mN/m at day 1 of fermentation. The ST reduction for MSM + HH + CH was 26.8 mN/m. After one day, ST reached a minimum of 26.3 mN/m. Considering these results, it can be inferred that the strain ZSB10 can use both cellulosic and hemicellulosic hydrolyzates to produce biosurfactants. In all prior cases, the surface tension reduction was higher when MSM was used as culture broth for biosurfactant production, because it only was observed a reduction of △ST = 50–32 = 18 mN/m. In all cases, the production of extracellular biosurfactant was evidenced since a substance can be considered as biosurfactant when added to distilled water is able to lower the ST in more than 8 units (Rivera et al., 2007; Rodríguez-Pazo et al., 2013).

On the other hand, cell-bound biosurfactant was obtained by washing out of biomass with distilled water. The ST of water (72 mN/m) was considered at day 0 to evaluate the surface tension reduction. Fig. 2b shows the strong reduction in ST on biosurfac-

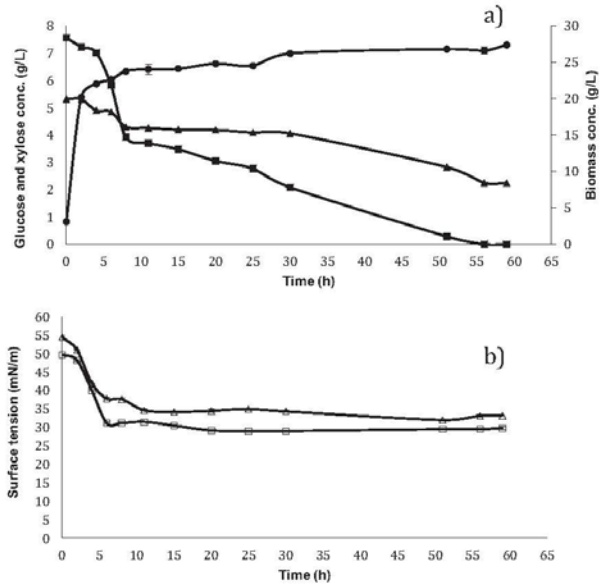

Fig. 4. Biosurfactants production by the strain ZSB10 using the culture medium MSM + HH + CH (mineral salt medium with the mixture of hemicellulosic (50%) and cellulosic (50%) hydrolyzates) in a 2-L bioreactor starting with an initial biomass concentration of 3.33 g/L. Incubation conditions: 35 °C, 150 rpm, and pH 7.4. Glucose consumption (■); xylose consumption (▲); biomass (●); surface tension of extracellular biosurfactants (□); surface tension of cell-bound biosurfactants (△).

tants obtained from biomass of all culture media. Such reduction was higher at day 1 in most cases. An analysis of variance with the results of the ST of day 1 showed significant difference ($p < 0.05$), however, among some culture media there was no significant difference ($p > 0.05$).

The lowest ST value of cell-bound biosurfactants was achieved using the MSM + CH medium (ST = 30 mN/m), meanwhile the highest ST value was obtained with CH medium (ST = 35.8 mN/m). It is noticeable that the latter showed the lowest surface tension value using extracellular biosurfactant.

Comparing all these previous results measured with extracellular and cell-bound biosurfactants, it can be concluded that extracellular biosurfactants have better surfactant characteristics achieving the lowest values of ST. Rodríguez-Pazo et al. (2013), using co-cultures of *Lactobacillus plantarum* and *Lactobacillus pentosus*, reported a surface tension reduction of only 17.2 mM/m under conditions where only cell-bound surfactants were present. Similarly, Moldes et al. (2007), using *L. pentosus* grown in hemicellulosic hydrolyzates, reported a surface tension reduction of 21 mM/m.

Furthermore, the consumption of sugars by strain ZSB10 was monitored in Fig. 1b (glucose) and Fig. 1c (xylose) at times 0, 24 and 48 h. HH + CH (50% hemicellulosic and 50% cellulosic) culture medium was mainly considered in order to obtain full use of both fractions. In this case, the initials glucose and xylose concentrations were 10.83 g/L and 5.11 g/L respectively. The microorganism consumed all the glucose, remaining 3.7 g/L of xylose after 24 h, which was further consumed after 48 h. On the other hand, MSM + HH + CH culture medium presented initials glucose and xylose concentrations of 9.69 g/L and 4.24 g/L respectively. The addition of MSM to the mixture of hydrolyzates affected its behavior remaining unconsumed residual amounts of sugars (0.19 g/L of glucose and 1.3 g/L of xylose) after 24 h, although these were completely consumed at the end of fermentation (48 h) meaning that there was neither an excess nor a deficit of nutrients due to the MSM addition. In general, the consumption of glucose by the microorganism was higher than consumption of xylose. It should be noted that glucose was present in higher concentration at time 0 regarding xylose.

3.2. Scaling up biosurfactants production using the hemicellulosic and cellulosic fractions of hydrolyzates in a 2-L bioreactor

The production of biosurfactants by strain ZSB10 was scaled up using the culture medium MSM + HH + CH (mineral salt medium with the mixture of hemicellulosic (50%) and cellulosic (50%) hydrolyzates), in order to supply nutrients from MSM medium and take advantage of both hydrolyzates of vine-trimming shoots. Previous assays revealed that biosurfactants obtained from MSM + HH + CH culture medium showed good results in the surface tension reduction (Fig. 2a and b).

Fig. 3a shows the growth of the strain ZSB10 in this medium, noticing a significant increase of biomass at 2 h (3.4 g/L) and 4 h (4.2 g/L) of incubation ($p < 0.05$), but continues growing slightly, until reaching a concentration of 4.5 g/L at 24 h and 5.0 g/L at the end of fermentation (52 h). Fig. 3a also describes the consumption of sugars (glucose and xylose) from the mixture of cellulosic and hemicellulosic vine-trimming shoots hydrolyzates supplemented with MSM during 52 h. The initial sugar concentration was of 8.3 g/L glucose and 5.1 g/L xylose.

Glucose was almost depleted at the end of fermentation (91.3%), however, xylose was scarcely consumed (30.8%) after 52 h. These results performed using 1 L of culture medium in a 2-L bioreactor are worse than those attained with the same experiment performed

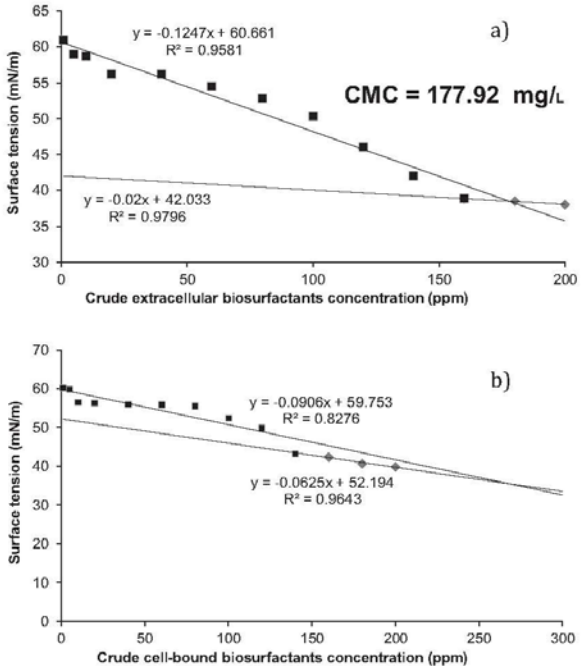

Fig. 5. Determination of critical micelle concentration (CMC) obtained from (a) crude extracellular biosurfactant and (b) crude cell-bound biosurfactant, obtained from fermentation of ZSB10 strain in MSM + HH + CH medium.

at a flask level (50 mL of medium in 250 mL Erlenmeyer flask), both inoculated with the same amount of biomass, where the substrate consumption was faster and higher. This poor performance cannot be attributed to pH, since this value only decreased slightly during fermentation from 7.4 at time 0 to 6.24 after 52 h, due to the increase of the concentration of two acids: acetic acid that rose from 0 to 0.84 g/L, but mainly to the appearance of lactic acid at 20 h of fermentation, which was augmenting its concentration until 4.0 g/L at the end of fermentation.

Regarding biosurfactants production, Fig. 3b shows the decrease in ST produced by extracellular and cell-bound biosurfactants. There was a reduction in ST at time 0 due to the components of the medium. However, as the fermentation proceeded, the decrease in ST was attributed to the biosurfactants production. It should be noted that the extracellular biosurfactant fermented medium reached the lowest ST value after 6 h, and no significant change ($p > 0.05$) turn out between 6 h (28.2 mN/m) and 52 h of fermentation (28 mN/m) indicating that it was achieved the critical micelle concentration (CMC). The CMC is defined as the concentration of surfactant required to initiate the formation of micelles. When the CMC is reached, the ST does not continue to decline even adding more surfactants (Shavandi et al., 2011). Consequently, measuring the ST of the fermented culture medium does not enable the determination of the quantity of biosurfactants produced. Expressed as surface tension reduction (ΔST), extracellular biosurfactant resulted in a maximum reduction of 23.8 mN/m at 6 h, without significant differences ($p > 0.05$) between 6 and 52 h of fermentation.

Fig. 3b also shows the decrease in ST of water containing the extracted cell-bound biosurfactants. It was observed a slight decrease in ST of water at time 0 (when still there was no growth of biomass) from 72 mN/m to 61.5 mN/m due to dissolved salts in the supernatant. In this case, it was reached an important lowering in ST values during fermentation (ST = 30 mN/m, at 52 h) but these were not better than those obtained using extracellular biosurfactant.

3.3. *Scaling up biosurfactants production using the hemicellulosic and cellulosic fractions of hydrolyzates in a 2-L bioreactor increasing the concentration of the initial inoculum*

The initial concentration of inoculum was increased 10 times (3.33 g/L dry weight biomass), with the purpose to determine if there was an improvement in the production and activity of biosurfactant with a larger initial biomass concentration.

Fig. 4a shows the faster growth of ZSB10 strain in this medium, noticing a significant increase ($p < 0.05$) during the first 2 h (20.2 g/L) being slower thereafter until reaching a final concentration of 27.4 g/L at 59 h. Additionally, the consumption of sugars (glucose and xylose) from the hemicellulosic and cellulosic vine-trimming shoots hydrolyzates was also monitored, in this way in Fig. 4a can be observed that glucose was completely depleted, however, only 57.63% xylose was consumed in the same period of time.

In comparison with the previous experiment, performed with a smaller amount of biomass, it can be concluded that when the initial biomass was higher there was an increase in the utilization of glucose with a higher rate of glucose consumption. Regarding

xylose consumption, there were no big differences between experiments. In both cases the consumption of xylose occurred when the glucose was being diminished. Additionally, during fermentation occurred a small change of pH from 7.07 to 6.26 at 59 h, which can be attributed to the production of lactic and acetic acids after 11 h of fermentation, ending with concentrations of 2.1 and 1.6 g/L, respectively.

On the other hand, Fig. 4b shows the values of ST measured on the culture medium with time. Notably, the lowest ST of extracellular biosurfactant (29.2 ± 0.3 mN/m) was reached at 20 h of fermentation and no significant changes ($p > 0.05$) were observed until the end of fermentation (29.8 mN/m) meaning that CMC was reached after that time. The slight increment in ST values observed after 51 h could be due to the biosurfactants began to be consumed by the microorganism as substrate. Compared to the previous experiment, it could be observed that a faster decrease of ST and a better result (28.2 mN/m at 6 h) was obtained using a lower initial biomass concentration (0.33 g/L).

Finally, the measure of the ST in water containing the cell-bound biosurfactants showed a reduction in ST from 54.5 mN/m at time 0 to 37.8 mN/m after 6 h being almost constant from 11 h until achieving a lowest level of 32 mN/m after 51 h. ST results of cell-bound biosurfactant are slightly superiors to those achieved in the previous experiment with smaller amount of initial biomass, where the lowest ST (30 mN/m) was achieved at 52 h of fermentation. These results seem contradictory, since having more biomass should have more cell-bound biosurfactant since the metabolite is found inside or attached to the cells. It is feasible that during fermentation this cell-bound biosurfactant became extracellular due to shear in the bioreactor.

3.4. Extraction and determination of critical micelle concentration (CMC) of crude biosurfactants

The culture medium MSM + HH + CH (mineral salt medium with the mixture of hemicellulosic (50%) and cellulosic (50%) hydrolyzates) fermented in a 2-L bioreactor, starting with an initial biomass concentration of 0.33 g/L, was used for crude biosurfactants extraction.

Crude extracellular biosurfactant had a yield of 1.52 g/L and lowered ST to 38.6 mN/m with a CMC = 177.92 mg/L (Fig. 5a). However, crude cell-bound biosurfactant only had a yield of 0.0783 g/L with a ST value of 39.8 mN/m as it is shown in Fig. 5b where it was not possible to determine the CMC of cell-bound biosurfactant because the experiment was carried out with a maximum concentration of 200 mg/L and this amount was not enough since in Fig. 5b did not occur the intersection of lines that is required for CMC determination.

Compared with those yields reported in literature, Rivera et al., (2007) had an intracellular biosurfactant yield of 0.0048 g/L using *L. pentosus* and grape marc, an useless agricultural residue from the wine industry, as substrate. Rodríguez et al., (2010) used wine industry waste materials such as vine-trimming shoots and distilled wine lees (vinasses) as culture media for biosurfactants production by *L. lactis*, the biosurfactant yield obtained was 0.0017 g/L and 0.0015 g/L expressed as surfactin equivalent, by two different methods. Using vine trimming shoots and a microbial co-culture of *L. plantarum* and *L. pentosus*, Rodríguez-Pazo et al., (2013) reported a maximum cell-bound biosurfactant yield of 0.0056 g/L. Consequently, the yield of extracellular crude biosurfactant produced by ZSB10 strain, using a similar culture medium, overcame these results.

Furthermore, in spite of the relatively high CMC values achieved by the strain ZSB10, the ST was decreased below 40 mN/m. The CMC value of extracellular biosurfactant resulted to be high, however, reports have been found using partially purified biosurfactants with higher CMC values than the reported in this work. For example Bodour et al., (2004) worked with a biosurfactant that diminished the ST value to 26 mN/m with a CMC value of 300 mg/L. Chen et al., (2012) used a biosurfactant produced by *B. licheniformis* TKU004 that decreased ST from 72 mN/m to 25.4 mN/m with a high CMC value of 350 mg/L.

Finally, E_{24} of crude biosurfactants indicated that both extracellular and cell-bound biosurfactants were able to emulsify kerosene, with E_{24} values of 47% and 41% respectively. Although both biosurfactants showed ability to emulsify kerosene, the emulsion of extracellular biosurfactants was more stable after 24 h in repose.

4. Conclusion

The strain *B. tequilensis* ZSB10 was able to consume hemicellulosic and cellulosic sugars obtained by sequential treatments of vine-trimming shoots to produce extracellular and cell-bound biosurfactants, in the presence or absence of salts. These results confirmed that ZSB10 strain is halotolerant. The process was scaled up from Erlenmeyer flask to 2-L bioreactor consuming effectively the mixture of hydrolyzates. Crude extracellular biosurfactants had a yield of 19.4 times the value achieved with crude cell-bound biosurfactant. Comparing the ST and CMC values between both kinds of biosurfactants, extracellular biosurfactant had better surfactant characteristics than cell-bound biosurfactant. Nevertheless, both biosurfactants showed ability to emulsify kerosene, although, the emulsion was more stable using extracellular biosurfactant after 24 h in repose.

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