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AVALIAÇÃO DO METABOLISMO DE *LACTOBACILLUS VINI* EM RESPOSTA À COMPOSIÇÃO NUTRICIONAL DO MEIO

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Área de concentração: Biotecnologia

Orientador: Prof. Dr. Marcos Antonio de Morais Júnior

Coorientador: Prof. Dr. Rafael Barros de Souza

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RESUMO

Lactobacillus vini é uma bactéria recentemente descrita e encontrada principalmente em ambientes industriais de fermentação. No Brasil e na Suécia esta bactéria foi encontrada na produção de etanol combustível como uma das principais contaminantes do processo industrial. Apesar de sua relevância, não há ainda uma análise consistente de sua fisiologia que esclareça a característica adaptativa a tais ambientes. O presente trabalho teve como objetivo a identificação de fatores nutricionais que podem explicar a presença de *L. vini* na fermentação industrial que utiliza derivados da cana-de-açúcar como substrato. Os resultados obtidos permitem concluir que, no processo de fermentação industrial, *L. vini* metaboliza glicose, sacarose e frutose, apenas com o objetivo de produzir ATP, através da produção exclusiva de ácido lático, e que utiliza os recursos orgânicos disponíveis no meio ambiente, como oligopeptídeos e aminoácidos, para geração de biomassa. Portanto, o crescimento de *L. vini* no meio industrial parece mais limitado pela composição orgânica do substrato. Em adição, as análises metabólicas mostraram que esta bactéria assimila citrato e acetato basicamente como fonte de carbono para geração de biomassa. As análises de linhagens mutantes indicaram que os genes relA e prcR parecem desempenhar papeis biológicos diferentes no metabolismo de carboidratos identificados em outras bactérias. Por fim, a capacidade de *L. vini* JP7.8.9 em fermentar dissacarídeos como sacarose e celobiose, bem como lactose, com elevados rendimentos em ácido lático torna essa bactéria uma candidata a aplicação em processos industriais

Palavras-chave: Bactérias lácticas. Caldo de cana. Citrato. Fermentação alcoólica.

ABSTRACT

Lactobacillus vini is a bacterium recently described and found mainly in industrial fermentation environments. In Brazil and Sweden, this bacterium was found in the production of fuel ethanol as one of the main contaminants of the industrial process. Despite this relevance, there is not a consistent analysis of its physiology that provides the adaptive characteristic to such environments. In this context, the present work aimed to identify nutritional factors that may explain the presence of *L. vini* in industrial fermentation that uses sugarcane derivatives as a substrate. The results allow us to conclude that, in the industrial fermentation process, *L. vini* metabolizes glucose, sucrose and fructose, with the sole purpose of producing ATP, through the exclusive production of lactic acid, and utilizing the organic resources available in the environment, oligopeptides and amino acids for biomass generation. Therefore, the growth of *L. vini* in the industrial environment seems more limited by the organic composition of the substrate. In addition, metabolic analyzes have shown that this bacterium assimilates citrate and acetate primarily as a carbon source for biomass generation. Analysis of mutant strains indicated that relA and prcR genes appear to play different biological roles in carbohydrate metabolism identified in other bacteria. Finally, the ability of *L. vini* JP7.8.9 to ferment disaccharides such as sucrose and cellobiose, as well as lactose, with high yields of lactic acid makes this bacterium a candidate for application in industrial processes.

Keywords: Lactic acid bacteria. Sugarcane juice. Citrate. Alcoholic fermentation.

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LISTA DE ABREVIATURAS E SIGLAS

ABC	Cassete de ligação de ATP (ATP-binding cassette)
ATCC	Coleção de cultura americana (American Type Culture Collection)
ATP	Adenosina trifosfato (Adenosine Triphosphate)
BAL	Bactéria ácido lática
CcpA	Proteína A de controle catabólico (Catabolite Control Protein A)
CitMHS	Fanília de proteínas responsáveis pelo transporte secundário de complexos citrato-metal em bactérias
CitH	Transportador de citrado-proton
CitM	Transportador do complexo citrado-magnésio
CitP	Citrato permeasse
CitS	Transportador do complexo citrado-sódio
CL	Citrato Liase
DHAP	Dihidroxiacetona fosfato (Dihydroxyacetone phosphate)
EMP	Via Embden-Meyerhoff-Parnas
FDP	Frutose-1,6-difosfato (Fructose 1,6-diphosphate)
FHE	Heterofermentativo Facultativo (Facultative Heterofermentative)
GAP	Gliceraldeído-3-fosfato (Glyceraldehyde 3-phosphate)
GC	Guanina+Citosina
GDB248	Linhagem Industrial de *Dekkera bruxellensis*
GlcU	Glicose permease
GPH	Glicosídeo-pentosideo-hexuronídeo (transportador)
GTP	Guanosina tri-fosfato (Guanosine triphosphate)
HPLC	Cromatografia liquida de alta pressão (High-performance liquid chromatography)
HPr	Proteína fosfocarreadora

JP1	Linhagem industrial de *Saccharomyces cerevisiae*
JP 7.8.9	Linhagem industrial de *Lactobacillus vini*
LAG	Fase latente do crescimento microbiano
LDH	Lactato desidrogenase
Mont4	Linhagem tipo de *Lactobacillus vini*
OAD	Oxaloacetato descarboxilase
OHE	Heterofermentativo Obrigatório (Obligately Homofermentative)
OHO	Homofermentativo Obrigatório (Obligately Homofermentative)
P-Ser-HPr	Proteína co-repressora do mecanismo de repressão catabólica
PE-2	Linhagem Industrial de *Saccharomyces cerevisiae*
PEP	Fosfoenolpiruvato (Phosphoenolpyruvate)
pH	Potencial Hidrogeniônico
PK	Via Fosfocetolase (Phosphoketolase Pathway)
PP	Via Pentose Fosfato (Pentose Phosphate Pathway)
PTS	Sistema fosfotransferase (Phosphotransferase system)

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1 INTRODUÇÃO

A produção de etanol combustível do Brasil tem como principal matéria-prima utilizada a cana-de-açúcar (BASSO et al., 2011; CASTRO et al., 2018), cujos derivados caldo e melaço são fermentados de maneira asséptica. A reutilização da biomassa celular ao longo de vários reciclos de fermentação é uma prática comum que reduz a necessidade de reprodução da população celular (BASSO et al., 2011). Essas práticas favorecem uma constante dinâmica populacional que possibilita uma série de contaminações recorrentes por leveduras e bactérias. Isto ocasiona instabilidade da população de *Saccharomyces cerevisiae,* levedura mais empregada no processo e de maior capacidade fermentativa quando comparada a outros microrganismos, e interfere no rendimento e produtividade da fermentação industrial, seja pela competição com a levedura pelo açúcar do meio, seja pela produção de compostos, como o lactato e o acetato, que diminuem a viabilidade das leveduras (DE OLIVA-NETO; YOKOYA, 1994; NARENDRANATH et al., 1997; BASSO et al., 2014). Espécies do gênero *Lactobacillus* se destacam como principais contaminantes e, dentre essas, a espécie *Lactobacillus vini* tem papel relevante em fermentações de destilarias do Nordeste do Brasil (LUCENA et al*.,* 2010). Inicialmente detectada na França, em mosto fermentado de uva para produção de vinho, a linhagem Mont 4 foi identificada apenas ao nível de gênero (BARRE, 1978), e mais tarde compôs juntamente com linhagens isoladas de vinho Espanha como a nova espécie *L. vini,* sendo então considerada a linhagem tipo da espécie (RODAS et al., 2005; RODAS et al.2006). Devido a sua presença em plantas de produção de etanol em destilarias na Suécia (PASSOTH et al*.,* 2007) e no Brasil (LUCENA et al*.,* 2010), estudos foram realizados no sentido de se identificar sua relação com leveduras (*S. cerevisiae* e *Dekkera bruxellensis*) comumente encontradas no processo industrial e compreender os aspectos que lhe permitem permanecer no processo (DE SOUZA et al*.,* 2012).

O presente trabalho é a parte de um conjunto de estudos realizados sobre a bactéria *Lactobacillus vini*, desde a sua identificação como contaminante da fermentação alcoólica industrial em destilarias do Nordeste do Brasil (LUCENA et al., 2010). Esses estudos já realizados compreendem avaliação da sua influência na capacidade fermentativa e interação com leveduras do processo industrial (DE SOUZA et al 2012); sequenciamento do genoma (LUCENA et al., 2012) e identificação de mecanismos moleculares de resistência a antibióticos e de tolerância a acidificação do meio (MENDONÇA et al., 2016; 2019). Portanto, o presente trabalho busca aprofundar os conhecimentos sobre a fisiologia de *L. vini* em resposta a composição nutricional do meio industrial.

Nesse contexto, este trabalho teve como principal objetivo analisar o metabolismo e fisiologia da linhagem industrial *Lactobacillus vini* JP7.8.9, isolada da destilaria Japungu, localizada no Nordeste de Brasil, para entender sua grande adaptabilidade ao ambiente fermentativo. No primeiro capítulo estão apresentados dados sobre a influência da composição nutricional do caldo e do melaço de cana sobre o crescimento celular e o papel do citrato na geração de energia e biomassa. No segundo capítulo são discutidos os dados relativos à utilização dos diferentes tipos de açúcares presentes nos substratos industriais sobre o crescimento celular e a influência dessas fontes de carbono sobre a capacidade homofermentativa da bactéria na produção de lactato.

1.1 Objetivos

1.1.1. Objetivo geral

- Determinar o perfil metabólico e fisiológico da linhagem industrial *L. vini* JP7.8.9 em meios de cultura sintéticos e industriais

1.1.2. Objetivos específicos

- Avaliar a influência da composição nutricional do meio na fisiologia da linhagem *L. vini* JP 7.8.9
- Analisar o perfil metabólico de *L. vini* em resposta as principais fontes de carboidratos presente no meio industrial.
- Avaliar a co-assimilação dos principais açúcares de interesse industrial por *L. vini* JP 7.8.9
- Avaliar o metabolismo de lactose em *L. vini* JP 7.8.9

2 REVISÃO DE LITERATURA

2.1 A produção de etanol

A fermentação alcoólica é um processo biológico que consiste na metabolização de açúcares fermentáveis, que são convertidos em energia celular, tendo como principal produto de interesse o etanol, que é o biocombustível mais consumido no mundo (BASSO et al*.,* 2011; CASTRO et al*.,* 2018). A utilização do etanol no Brasil, como biocombustível encontrou seu ápice na década de 70 com a criação do programa Proálcool (Programa Nacional do Álcool), que surgiu devido à crise internacional do petróleo, iniciando assim uma política de incentivo ao uso e produção do etanol como combustível, com aumento de investimentos no setor (BASSO et al., 2011; CASTRO et al., 2018). Além disso, a maioria dos combustíveis líquidos do mundo são derivados do petróleo que, além de não ser renovável, contribui com o aquecimento global devido à grande emissão de gases de efeito estufa, gerados pelo seu uso, o que fez com que se intensificasse a procura por um substituto que pudesse ser produzido com baixo custo, a partir de fontes renováveis e que pudesse ser produzido em grande escala (CASTRO et al*.,* 2018). A produção de bioetanol representa uma tecnologia em grande escala capaz de produzir biocombustíveis de forma eficiente economicamente, oferecendo substitutos viáveis à gasolina (AMORIM et al., 2011; CASTRO et al., 2018). Há várias décadas certos microrganismos exercem uma forte importância biotecnológica como, por exemplo, a levedura *Saccharomyces cerevisiae* que, de forma eficiente, é capaz de assimilar diversas fontes nutricionais do caldo de cana de açúcar e produzir etanol (WALKER, 2003), conseguindo altos rendimentos na sua produção devido à sua alta capacidade fermentativa, sendo amplamente empregada nesse tipo de processo. O Brasil é o país que introduziu pela primeira vez esse combustível renovável em sua matriz energética (BASSO et al., 2011), sendo a produção brasileira, a partir da cana-de-açúcar, um dos maiores processos biotecnológicos industriais do mundo (BASSO; LINO, 2018).

O processo de fermentação alcoólica nas destilarias brasileiras vem sendo fonte de vários estudos sobre a dinâmica populacional tanto de leveduras como de bactérias, principalmente por se tratar de um ambiente não estéril e, portanto, propício ao aparecimento de diferentes microrganismos considerados contaminantes (BASSO et al., 2011; Basso et al., 2014; BASSO; LINO, 2018). Apesar do aparecimento de microrganismos selvagens, sejam eles leveduras ou bactérias, geralmente oriundos do caldo de cana, algumas linhagens de *S. cerevisiae* são capazes de se estabelecer ao longo da safra. Essas linhagens podem variar de acordo com a região, como por exemplo, a linhagem *S. cerevisiae* PE-2 que permanece dominante em 58% das destilarias do estado de São Paulo (BASSO et al., 2008), e a linhagem JP1 que é mais adaptada as características da região nordeste (SILVA-FILHO et al., 2005a). Ambas, além de permanecerem no processo apresentam uma alta produção de etanol e eficiência de fermentação, fazendo com que se tornem comercialmente muito atrativas no setor sucroalcooleiro.

Outras espécies de leveduras também são capazes de se estabelecer no processo, porém, não tem a mesma capacidade fermentativa e se tornam indesejadas, como por exemplo, a levedura *Dekkera bruxellensis* (LIBERAL et al., 2007; BASÍLIO et al., 2008). Esta levedura também é relatada na indústria do vinho e da cerveja por produzirem compostos tanto desejáveis como indesejáveis às bebidas (BOULTON et al., 1996). Essa espécie vem sendo estudada ao longo dos anos, principalmente, para entender suas habilidades de adaptação as condições industriais (CIANI et al., 2003; PEREIRA et al., 2011; DE BARROS PITA et al., 2011, 2013; DE SOUZA et al., 2012; LEITE et a*l*., 2013), mas também para uma possível utilização dessa levedura na produção de etanol em consórcio com outros microrganismos (PASSOTH, et al., 2007) e também na produção de cachaça (PARENTE et al., 2014).

Essas contaminações por microrganismos selvagens, ou seja, microrganismos que não faziam parte da microbiota inoculada no processo, podem causar danos significativos na produção de etanol (AMORIM et al*.,* 2004; SILVA-FILHO et al., 2005b**;** BASSO et al*.,* 2014) pois interferem negativamente no desempenho fermentativo das leveduras do processo, seja competindo por açúcares fermentáveis e nutrientes do meio, seja pela produção de produtos, como ácidos orgânicos, causando diminuição no rendimento em etanol, floculação das células de levedura e a baixa viabilidade das leveduras (DE OLIVA-NETO; YOKOYA, 1994; NARENDRANATH et al*.,* 1997; BASSO et al., 2014). Portanto, o acompanhamento da microbiota tanto da matéria-prima, como também da fermentação se torna essencial para um devido controle do processo.

2.2 Contaminação Bacteriana

Tendo em vista a natureza do processo e os grandes volumes processados, a contaminação bacteriana é um problema recorrente nas fermentações industriais (BASSO; LINO, 2018). Além de ser um processo aberto, onde os substratos (melaço e/ou caldo de cana) não são esterilizados, a produção industrial envolve, por muitas vezes, o reciclo do fermento e, consequentemente, o reciclo dos contaminantes. Estas condições tornam o ambiente complexo e susceptível a uma constante variação de diferentes espécies e linhagens de leveduras e bactérias ao longo do período de fermentação, o que pode causar diversos distúrbios no processo, como a diminuição da produtividade além de outros problemas operacionais (AMORIM et al*.,* 2004; SILVA-FILHO et al., 2005b). As bactérias competem com as leveduras do processo por diversos nutrientes e, principalmente, pelos açúcares, que são a principal fonte de carbono (NARENDRANATH et al., 1997; MUTHAIYAN et al., 2011) e que, ao ser consumido por elas, são convertido em outros subprodutos como lactato e/ou acetato, em detrimento da produção de etanol (INGLEDEW, 1995; NARENDRANATH, 2003). Esses ácidos orgânicos podem, ainda, ocasionar inibição do crescimento e do metabolismo das leveduras (NARENDRANATH et al., 2001; NARENDRANATH, 2003; SKINNER, 2004; BASSO et al*.,* 2014). As bactérias podem ainda provocar a floculação das leveduras, que ocasiona redução na velocidade da fermentação, além de inconvenientes como entupimento de tubulações, aumento de fundo da dorna, dificuldade de operação das centrífugas devido ao entupimento dos bicos (SERRA et al*.,* 1979; YOKOYA; DE OLIVA-NETO, 1991) e impossibilitar o reciclo do fermento.

Além disso, na fermentação de vinho, a presença excessiva de bactérias pode causar sérios problemas, devido ao aumento da acidez do produto final e a diminuição da viabilidade das células de leveduras (YOKOYA; OLIVA-NETO, 1991). Tudo isto gera custos para a indústria, que vão desde a limpeza das dornas, utilização de antibióticos, à troca do fermento e queda na eficiência e no rendimento da fermentação**,** fazendo desta uma das questões mais relevantes economicamente na produção de etanol (MUTHAIYAN et al., 2011; BASSO et al., 2014).

Algumas bactérias Gram-negativas costumam aparecer na plantas de produção, como as pertencentes aos gêneros *Acetobacter e Gluconobacter,* que produzem ácido acético como principal subproduto de seu metabolismo. Apesar disso, as Gram-positivas compreendem os grupos mais representativos (NARENDRANATH, 2003). As espécies pertencentes aos gêneros *Lactobacillus, Leuconostoc, Pediococcus* e *Weissella* estão entre as espécies de bactérias láticas mais comumente encontradas na indústria de etanol, possivelmente, por serem tolerantes à baixos valores de pH, altas temperaturas e apresentarem ainda altas taxas de crescimento (NARENDRANATH et al., 1997). Entre estes grupos, os *Lactobacillus* predominam nos processos de fermentação, como tem sido observado por um longo tempo, sendo considerados os principais contaminantes na produção de etanol (KANDLER, 1983; KANDLER; WEISS, 1986; DE OLIVA-NETO; YOKOYA, 1994; CHANG et al*.,* 1995; SCHELL et a*l*., 2007; BECKNER et al*.,* 2011). Em estudo comparando o efeito de bactérias heterofermentativas

(*Lactobacillus fermentum*) e homofermentativa (*Lactobacillus plantarum*) no rendimento fermentativo foi constatado que a *L. fermentum* causa um maior efeito negativo na fermentação alcóolica em relação a *L. plantarum*, gerando uma diminuição no rendimento em etanol (BASSO et al., 2014). Estudos em destilarias do nordeste brasileiro revelaram uma grande diversidade de espécies de *Lactobacillus*, inclusive *L. vini*, tanto no caldo de cana de açúcar, como também no mosto fermentado (LUCENA et al., 2010).

2.3 Bactéria Láticas

Apesar da grande variedade de bactérias já detectadas na industria, incluindo espécies de *Acetobacter*, *Bifidobacterium, Clostridium, Enterococcus, Gluconobacter, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus* e *Weissella*, a maior parte das bactérias identificadas em plantas de produção de etanol pertence ao grupo das bactérias ácido-láticas (BAL) (CHANG et al., 1995; SKINNER, 2004; LUCENA et al., 2010).

Composto por cerca de 450 espécies o grupo das BAL é, historicamente, definido como um grupo heterogêneo (MOZZI, 2016), de microrganismos filogeneticamente relacionados e que tem o ácido lático como único ou principal produto da fermentação de carboidratos (LIU et al., 2014; SUN et al., 2014; MOZZI, 2016) (SUN et al., 2014). São bactérias Gram-positivas, não esporulantes, normalmente catalase-negativas, embora algumas cepas possam produzir pseudo-catalases, possuem forma de cocos, cocobacilos ou bacilos (KANDLER, 1983; AXELSSON, 2004; KÖNIG; FRÖHLICH, 2009; SUN et al., 2014), e contém baixo conteúdo de GC em seu DNA (KÖNIG; FRÖHLICH, 2009; SUN et al., 2014; HOLZAPFEL; WOOD, 2014) e apresentam um complexo requerimento nutricional (KANDLER; WEISS, 1986), requerendo aminoácidos, peptídeos, bases de nucleotídeos, vitaminas, minerais, ácidos graxos e carboidratos, componentes que geralmente estão presentes nos nichos naturais em que habitam (MOZZI, 2016). Podem assimilar uma variedade de nutrientes, fermentando os açúcares pela via Embdem-Meyerhoff-Parnas (EMP), onde o piruvato é convertido em ácido lático pela lactato desidrogenase (LDH), no caso das homofermentadoras, ou produzir outros compostos como, ácido acético, ácido fórmico, etanol e/ou dióxido de carbono, além do lactado, como as heterofermentadoras (KANDLER, 1983; HAMMES; VOGEL, 1995; HAMMES; HERTEL, 2009; KÖNIG; FRÖHLICH, 2009; VON WRIGHT; AXELSSON, 2012; SMID; KLEEREBEZEM, 2014; MOZZI, 2016).

Consideradas como um grupo onipresente (ADAMS, 1999), sua capacidade de habitar diferentes nichos é característica de sua diversidade metabólica e adaptabilidade (MOZZI, 2016). As BALs estão presentes em ambientes nutricionalmente ricos, podendo ser encontradas em diversos habitats como produtos alimentícios (lácteos, vegetais, carnes), material em decomposição e bebidas. São encontradas, ainda, em animais e humanos, em cavidades como a oral e vaginal, e tratos respiratório e gastrointestinal (KÖNIG; FRÖHLICH, 2009; VON WRIGHT; AXELSSON, 2012) onde são componentes comuns da microbiota comensal. Exceto por algumas espécies pertencentes aos gêneros *Streptococcus*, *Enterococcus*, *Lactococcus* e *Carnobacterium*, são não-patogênicos (MOZZI, 2016) e devido essa longa história de exposição e consumo humano chegou-se à conclusão razoável de que eles são geralmente seguros (ADAMS, 1999; MOZZI, 2016).

As BALs podem crescer em uma ampla gama de temperaturas, de 2˚C a 53˚ C, com uma temperatura ótima variando, geralmente, entre 30˚C e 40˚C. O pH ideal para o crescimento é de 5,5-6,0 e geralmente ocorre em pH 5,0 ou menos, mas a taxa de crescimento é significativamente reduzida. Em condições ideais de crescimento, o tempo de duplicação das bactérias lácticas é muito mais rápido do que o da levedura (NARENDRANATH, 2003). Essas bactérias são anaeróbicas e estritamente fermentativas, embora sejam aero-tolerantes (AXELSSON, 2004; KÖNIG; FRÖHLICH, 2009; VON WRIGHT; AXELSSON, 2012), e obtêm energia por fosforilação a nível de substrato, uma vez que não possuem um “sistema respiratório” funcional (LIU et al*.*, 2014). Apesar disso, várias de suas espécies podem mudar da fermentação para o metabolismo respiratório aeróbio quando os grupos heme ou heme e menaquinona são fornecidos (MOZZI, 2016).

São bactérias tolerantes a ácidos (KÖNIG; FRÖHLICH, 2009; Beckner et al*.*, 2011; SMID; KLEEREBEZEM, 2014; SUN et al., 2014; HOLZAPFEL; WOOD, 2014, MENDONÇA et al., 2019) e provocam uma diminuição no pH em associação com a produção de ácidos orgânicos, o que inibe o crescimento de microrganismos competidores (SMID; KLEEREBEZEM, 2014). Isto, aliado à sua permanência no trato gastrointestinal, pode possibilitar a utilização das BALs como probióticos, promovendo benefícios à saúde do hospedeiro. Seus efeitos benéficos na indústria de alimentos ainda incluem a habilidade de produzir compostos aromáticos e realçadores de sabor (CARR et al., 2002) fazendo com que sejam amplamente exploradas em inúmeras aplicações industriais, desde culturas iniciais na indústria de laticínios, vegetais e carnes, bem como no processamento de outros produtos como vinho (KONIGS et al., 2000).

Sendo consideradas importantes no que diz respeito à fermentação de alimentos e aplicações farmacêuticas (LIU et al*.*, 2014) as BALs têm sido extensivamente estudadas e os conhecimentos adquiridos abriram novas possibilidades para a sua aplicação. O redirecionamento dos fluxos metabólicos permite otimizar os processos de fabricação de alimentos, produzindo, inclusive, alimentos mais saudáveis ou para manipular as propriedades organolépticas (isto é, o sabor e textura global) bem como produção de produtos para aplicações no setor de saúde humana e veterinária (VENEMA et al., 1999).

2.3.1. Metabolismo dos açúcares

Fastidiosas, as BALs requerem meios ricos e complexos para um crescimento significativo, tendo os açúcares como a principal fonte de carbono e energia (KOWALCZYK et al., 2016; PESSIONE et al., 2010). Embora, de maneira geral, a glicose e a lactose sejam os açúcares preferenciais e mais amplamente utilizados por bactérias láticas (WU ; LU, 2014), outras hexoses também podem ser metabolizadas, bem como pentoses, dependendo de cada organismo. Apresentam, de forma geral, cinco vias principais de fermentação de carboidratos: as vias de Leloir e da Tagatose-6P (Figura 1), que são, ambas, vias para a metabolização da lactose e da galactose, a depender do sistema de transporte utilizado na internalização desses açúcares. Quando não fosforiladas, seguem pela Via de Leloir, e quando fosforiladas, seguem pela Via da tagatose-6P, via esta ligada à fosforilação da galactose. Quanto à glicose resultante da hidrólise da lactose, esta segue pela Glicólise (ISKANDAR et al., 2019); a Glicólise (Figura 2), ou via de Embden-Meyerhof-Parnas (EMP), que em condições de disponibilidade de açúcares e limitação de oxigênio, é caracterizada pela formação da frutose-1,6-difosfato (FDP), a partir de hexoses, que é quebrada pela FDP aldolase em duas trioses (DHAP – dihidroxiacetona fosfato e GAP – gliceraldeído-3-fosfato), que por sua vez, são convertidas a lactato (KANDLER, 1983; VON WRIGHT; AXELSSON, 2012); a Via das Pentoses-Fosfato (PP) (Figura 2), na qual as pentoses são fosforiladas e então convertidas a xilulose-5-fosfato e posteriormente em GAP pela ação das enzimas transcetolase e transaldolase, seguido pela conversão do GAP a piruvato, resultando em ácido lático. A via das pentoses pode ainda seguir pela Via Fosfocetolase (PK) (Figura 2), na qual a xilulose-5-fosfato, resultante de pentoses ou ainda da glicose-6-fosfato (quando esta é convertida a 6-fosfogluconato), sofre a ação da enzima fosfocetolase e é clivada em GAP e acetil-fosfato (Acetil-P), que são reduzidas, transformando-se em ácido lático e etanol e/ou ácido acético, respectivamente (KANDLER, 1983; KANDLER; WEISS, 1986; WANG et al., 2015).

Acredita-se que o metabolismo da glicose pela via EMP é a via catabólica primária, porém, algumas bactérias não apresentam, por exemplo, o gene *fba* que codifica a frutose-bifosfato-aldolase, enzima fundamental para esta via (MORITA et al., 2008). Durante a metabolização dos açúcares, bem como outros compostos, são gerados intermediários que podem ser utilizados pela célula como precursores em biossínteses e sustentam o crescimento celular (KIM; GADD, 2008), fazendo com que o catabolismo destes compostos seja de grande relevância para a geração de energia e biomassa (PESSIONE et al., 2010).

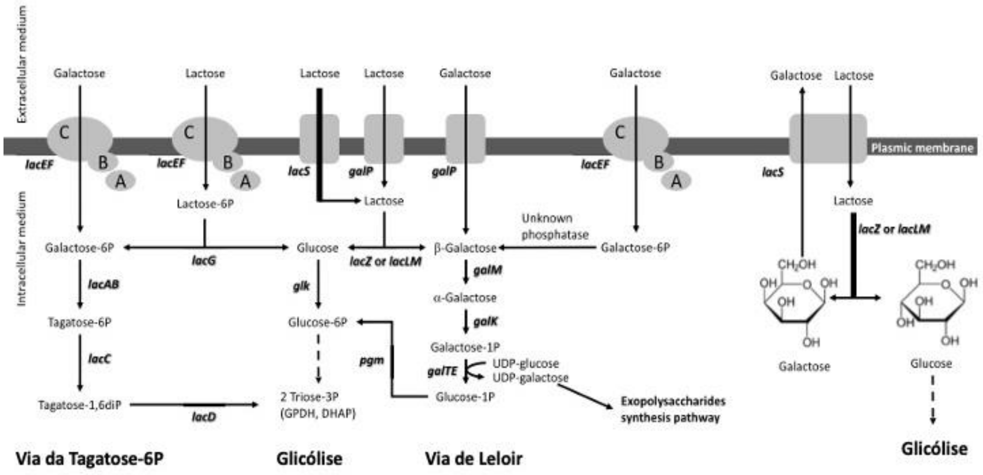
Extracellular medium
Intracellular medium
Galactose
Lactose
Lactose
Lactose
Galactose
Galactose
Galactose
Lactose
C
B
A
lacEF
lacEF
lacS
galP
galP
lacEF
lacS
Plasmic membrane
Lactose-6P
Lactose
Lactose
Unknown phosphatase
lacZ or lacLM
Galactose-6P
lacG
Glucose
lacZ or lacLM
β-Galactose
Galactose-6P
lacAB
glk
galM
α-Galactose
CH₂OH
OH
Tagatose-6P
Glucose-6P
galK
Galactose-1P
lacC
pgm
galTE
UDP-glucose
UDP-galactose
Galactose
Glucose
Tagatose-1,6diP
lacD
2 Triose-3P (GPDH, DHAP)
Glucose-1P
Exopolysaccharides synthesis pathway
Via da Tagatose-6P
Glicólise
Via de Leloir
Glicólise

Figura 1. Representação esquemática das vias metabólicas da lactose e da galactose. Enzimas codificadas pelos genes: lacAB, galactose-6-fosfato isomerase; lacC, tagatose-6-fosfato quinase; lacD, tagatose-1,6-difosfato aldolase; lacEF, EIIABClac / Gal; lacG, fosfo-β-galactosidase; lacR, regulador da transcrição; lacS, lactose permease; lacZ, β-galactosidase; lacLM, β-galactosidase; galP, galactose permease; galM, galactose epimerase; galK, galactose quinase; galT, galactose-1-fosfato uridiltransferase; galE, UDP-glucose-4-epimerase; pgm, fosfoglucomutase; glk, glucoquinase. GPDH: fosfato de gliceraldeído 3, DHAP: fosfato de dihidroxiacetona. A linha pontilhada simboliza várias reações. (Fonte: ISKANDAR et al*.*, 2019)

Estes açúcares podem ser internalizados para célula por diversos tipos sistemas de transporte, que podem ou não demandar de gasto energético incluindo, por exemplo, sistemas de Fosfotransferase (PTS), cassetes de ligação de ATP (ABC) e transportadores glicosídeo-pentosidoe-hexuronídeo (GPH) (MAYO et al*.*, 2010; KOWALCZYK et al*.*, 2016), uma vez que as BALs apresentam alto número de genes que codificam possíveis transportadores de açúcar. Os principais componentes para a catálise do transporte e fosforilação dos açúcares são os PTSs, dependentes de fosfoenolpiruvato (POSTMA et al., 1993) e que envolve o uso de fosfotransferase (KIM; GADD, 2008). O PTS catalisa a translocação de grupo, ou seja, o acoplamento do transporte de açúcar para a fosforilação deste, e o fosfoenolpiruvato é o doador inicial de fosforil. Ele fosforila a Enzima I e, em seguida, o grupo fosforila é sequencialmente passado para a proteína HPr, Enzima IIA, Enzima IIB e, finalmente, para um açúcar que entra em uma reação dependente do transportador transmembrana, Enzima IIC. As duas primeiras são inespecíficas e transportam qualquer açúcar. Os componentes Enzima II são específicos para cada açúcar particular (LORCA et al.*,* 2010; MADIGAN et al.*,* 2016) (Figura 3).

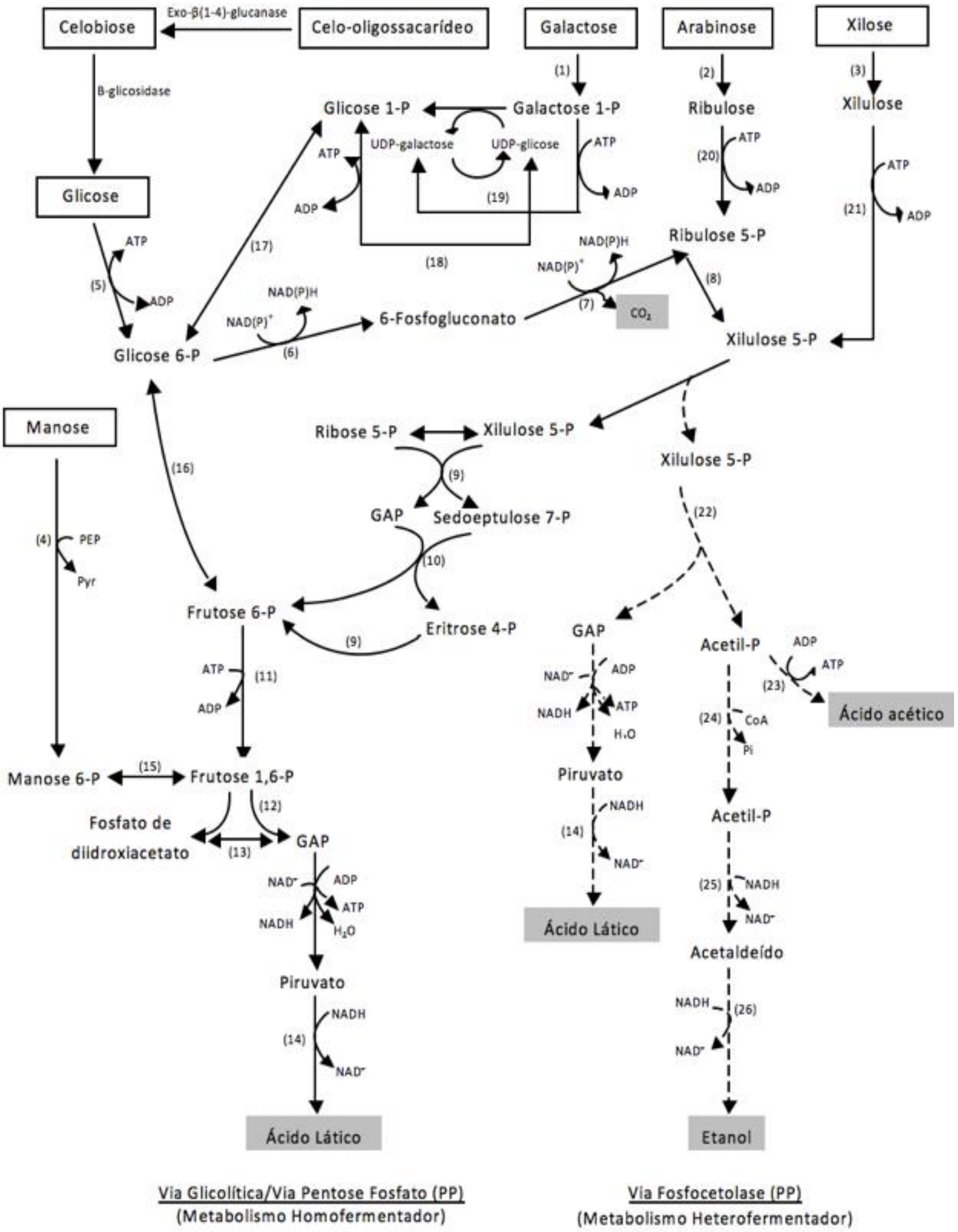
Celobiose
Exo-β(1-4)-glucanase
Celo-oligossacarídeo
Galactose
Arabinose
Xilose
B-glicosidase
(1)
(2)
(3)
Glicose 1-P
Galactose 1-P
Ribulose
Xilulose
UDP-galactose
UDP-glicose
ATP
ADP
(19)
(20)
(21)
Glicose
(18)
(17)
NAD(P)H
NAD(P)⁺
Ribulose 5-P
(7)
CO₂
(8)
(5)
6-Fosfogluconato
(6)
Glicose 6-P
Xilulose 5-P
Manose
Ribose 5-P
Xilulose 5-P
(9)
(16)
GAP
Sedoeptulose 7-P
(22)
(4)
PEP
Pyr
(10)
Frutose 6-P
Eritrose 4-P
(9)
GAP
Acetil-P
(11)
NAD⁺
NADH
H₂O
(23)
Ácido acético
(24)
CoA
Pi
Manose 6-P
(15)
Frutose 1,6-P
Piruvato
(12)
Fosfato de diidroxiacetato
(13)
GAP
(14)
Acetil-P
(25)
Ácido Lático
Acetaldeído
Piruvato
(26)
Ácido Lático
Etanol
Via Glicolítica/Via Pentose Fosfato (PP)
(Metabolismo Homofermentador)
Via Fosfocetolase (PP)
(Metabolismo Heterofermentador)

Figura 2. Vias para a produção de ácido láctico a partir de carboidratos, por parte de bactérias ácido láticas. GAP, gliceraldeído-3-fosfato; PEP, fosfoenolpiruvato; Pyr, piruvato. Enzimas: 1, galactoquinase; 2, arabinose isomerase; 3, xilose isomerase; 4, manose fosfotransferase; 5, 24exoquinase; 6, glicose-6-fosfato desidrogenase; 7, 6-fosfogluconato desidrogenase; 8, ribulose-5-fosfato 3-epimerase; 9, transcetolase; 10, transaldolase; 11, 6-fosfofrutoquinase; 12, frutose-bisfosfato aldolase; 13, triosefosfato isomerase; 14, lactato desidrogenase; 15, fosfomanose isomerase; 16, fosfoglucose isomerase; 17, fosfoglucomutase; 18, galactose-1-fosfato uridil-transferase; 19, glicosiltransferase; 20, ribulocinase; 21, xiluloquinase; 22, fosfocetolase;23, acetato quinase; 24, fosfotransacetilase; 25, aldeído desidrogenase; 26, álcool desidrogenase. (Fonte: WANG et al., 2015)

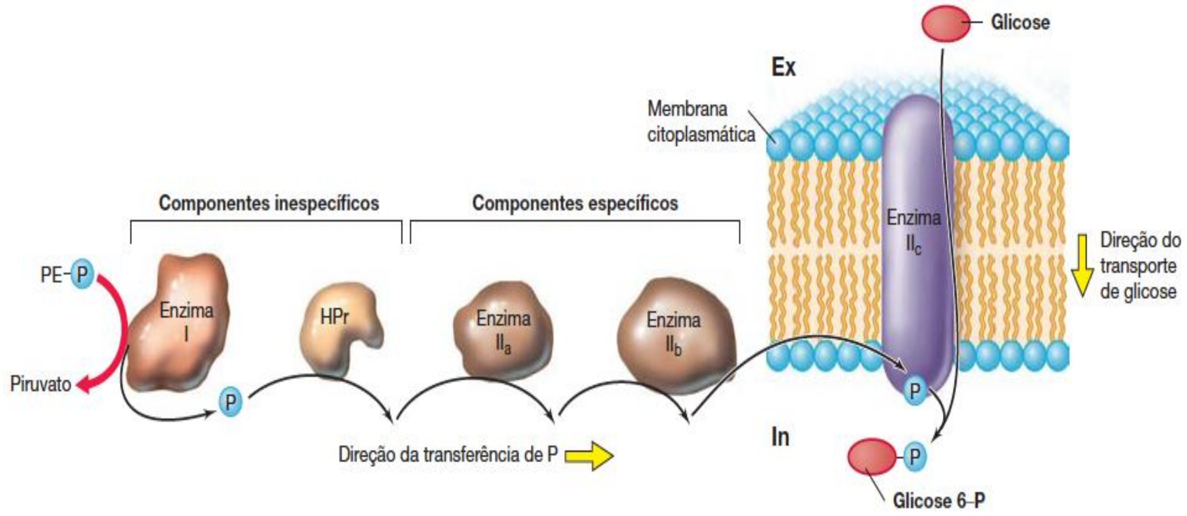
Glicose
Ex
Membrana citoplasmática
Componentes inespecíficos
Componentes específicos
Enzima II$_c$
Direção do transporte de glicose
PE-P
Enzima I
HPr
Enzima II$_a$
Enzima II$_b$
Piruvato
P
P
Direção da transferência de P
In
P
Glicose 6-P

Figura 3. Mecanismo do sistema fosfotransferase de *Escherichia coli.* (Fonte: Madigan *et al.,* 2016)

O número de genes relacionados aos sistemas PTS pode ter grande variação nas BALs, variando, inclusive, entre indivíduos da mesma espécie (ZHANG et al., 2013) e o número de proteínas PTS codificadas dentro do genoma destes indivíduos não se correlacionam, necessariamente, com o tamanho do genoma. Por exemplo, membros do filo Firmicutes têm a maioria das permeases de PTS, apesar de seu tamanho reduzido de genoma. Entretanto, nem todos os Firmicutes, incluindo algumas BALs, têm um grande número de carregadores de PTS (BARABOTE; SAIER, 2005). Além disso, os genomas de BAL contém muitos genes que codificam outros transportadores que estão envolvidos no transporte de fontes de carbono, como citado anteriormente. A maltose, por exemplo, é transportada para a célula através do sistema ABC (cassetes de ligação de ATP) (KIM; GADD, 2008), que, assim como o PTS, envolve gasto energético, e é composto em 3 componentes: uma proteína de ligação ao substrato que exibe alta afinidade pelo substrato a ser transportado; um transportador integrado à membrana, formando um canal de transporte; e uma proteína que hidrolisa ATP, fornecendo assim, energia ao sistema (MADIGAN et al*.,* 2016) (Figura 4). Existem ainda transportadores que não envolvem fosforilação das moléculas. Trata-se de transportes simples, que consistem somente em uma proteína transportadora transmembrânica, por onde a molécula de açúcar é transportada para o interior da célula por ação de uma força próton-motiva (MADIGAN *et al.,* 2016), como é o caso da permease não-PTS GalP, envolvida na importação da galactose (KOWALCZYK et al*.,* 2016), e a MelB, uma permeasse transportadora de melibiose, que pertence ao GPH, uma família de simporter de cátion (ETHAYATHULLA et al*.,* 2014).

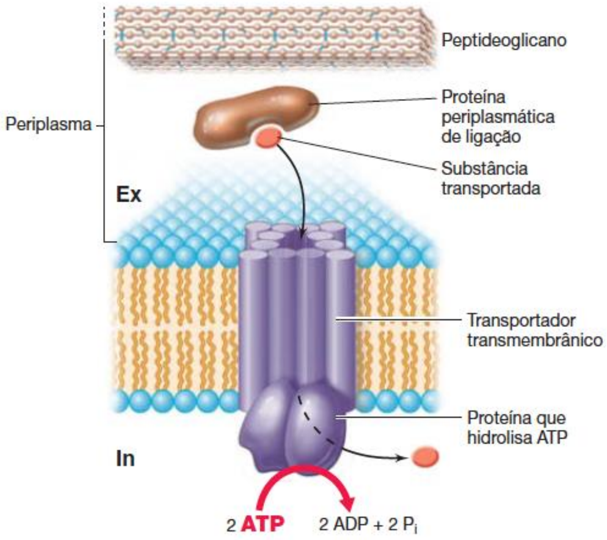
Peptideoglicano
Periplasma
Proteína periplasmática de ligação
Substância transportada
Ex
Transportador transmembrânico
Proteína que hidrolisa ATP
In
2 ATP
2 ADP + 2 P$_i$

Figura 4. Mecanismo de um transportador ABC. (Fonte: Madigan *et al.,* 2016) Madigan *et al.,* 2016)

Uma vez que o açúcar foi internalizado, as BAL empregam a homo ou heterofermentação, dependendo do sentido do fluxo metabólico. Os produtos finais primários da primeira via consistem principalmente de ácido láctico, enquanto a segunda produz ácido láctico, CO_2, ácido acético e, por vezes, etanol, de forma que se mantenha o balanço redox na célula. (KANDLER, 1983; KOWALCZYK et al*.,* 2016; ZHANG et al*.,* 2016). O perfil de assimilação de cada açúcar e o potencial fermentescível de cada espécie e/ou linhagem podem, entre outros fatores, ser influenciados pelo habitat, de forma que cada bactéria pode ter maior capacidade de assimilar açucares específicos disponíveis em abundância em seus ambientes naturais, indicando a sua adaptação a diferentes nichos ecológicos. *L. salivarius*, por exemplo, é considerado como homofermentativo, mas possui uma via fosfocetolase (do metabolismo heterofermentador) funcional (CLAESSON et al., 2006). Bactérias associadas à produção de laticínios podem metabolizar, com eficiência, a lactose. Já as associadas a plantas podem utilizar uma grande variedade de outros carboidratos, incluindo os β-glicosideos (ALEKSANDRZAK-PIEKARCZYK, 2013). Um exemplo disto é a bactéria lática *Lactobacillus vini,* que teve linhagens distintas encontradas no processo de produção de vinho na Espanha, como também em destilarias produtoras de bioetanol, no nordeste do Brasil. Embora estas linhagens apresentem grandes semelhanças no que diz respeito a um perfil “geral” de assimilação de açúcares, foram encontradas particularidades que diferem estas linhagens entre si, e as diferem também da linhagem tipo. Estas diferenças podem ser explicadas por uma provável adaptação ao ambiente (dados ainda não publicados), que, por sua vez, demanda uma adaptação no metabolismo celular.

2.3.2. Resposta Estringente

Na natureza além da capacidade de utilizar diversas fontes de carbono os microrganismos precisam ajustar seu metabolismo à disponibilidade nutricional (BOUTTE; CROSSON, 2013). De forma geral, o meio ambiente tende a ser bastante desfavorável, já que apresenta situações bastante adversas, como por exemplo alterações climáticas e limitada disponibilidade de nutrientes. A carência nutricional provoca alteração no metabolismo celular, e em diversos organismos esse controle tem sido dependente da produção de uma molécula conhecida com alarmônio produzida a partir de GTP (GEIGER; WOLZ, 2014). O acúmulo do alarmônio no citoplasma celular tem um efeito pleiotrópico nos organismos estudados. Em organismos modelo como *Escherichia coli* e *Bacillus subtilis*, os principais efeitos da resposta estringente envolve a desrepressão dos genes de fontes secundárias de carbono e da resposta geral a estresse (BATTESTI et al*.*, 2011; GEIGER; WOLZ, 2014).

A produção do alarmônio é dependente da proteína codificada pelo gene *relA* ou *spoT*, ambos desempenhando a mesma atividade enzimática (TRAXLER et al*.*, 2008). Embora esses genes não sejam essenciais para sobrevivência da célula, são indispensáveis para sobrevida da população durante condições adversas de crescimento (KASAI et al*.*, 2006; TRAXLER et al*.*, 2008). A resposta estringente está intimamente relacionada ao regulon do gene *codY* (GEIGER; WOLZ, 2014). Em condições de abundância nutricional a resposta estringente é desativada, a produção do alarmônio diminui e o *pool* de GTP volta a aumentar (SONENSHEIN, 2005). O aumento da produção de energia também contribui com o aumento da produção do GTP que passa a refletir a abundância energética da célula. O GTP juntamente com os aminoácidos leucina, isoleucina e valina, são moduladores positivos do regulador *codY* que leva a repressão dos genes de metabolismo secundário de carbono, de resposta geral a estresse e de outras características celulares de fase estacionária (SONENSHEIN, 2005). Deste modo, a resposta estringente e a ativação do regulon de *codY* estão diretamente implicados na regulação do metabolismo energético da célula e podem ser importantes componentes na sobrevivência dos *Lactobacillus* em ambientes adversos, como o ambiente industrial de produção de etanol.

2.3.3. Metabolismo do Citrato

Além dos açúcares, várias espécies de BAL têm a capacidade de metabolizar citrato, um processo que requer o transporte do citrato, sua possível conversão em oxaloacetato e depois em piruvato e CO_2 (MAGNI et al., 1999; MAYO et al., 2010). Entretanto, esse modelo carece de comprovação experimental e de análises metabólicas mais consistentes. Abundante na natureza, o citrato é uma importante fonte de energia para muitas bactérias (LAËTITIA et al., 2014) e vem sendo descrito na literatura por favorecer o crescimento de lactobacilos (MAGNI *et al.*, 1999; LAËTITIA et al., 2014). Sua metabolização pelas BALs pode levar à produção de lactato, formato, acetato e etanol (NEVES et al. 2005), bem como compostos voláteis como diacetil, acetoína e butanodiol (KOWALCZYK et al*.,* 2016; Neves et al., 2005) responsáveis pelo aroma típico de muitos produtos lácteos fermentados, e considerado off-flavors em outros produtos fermentados, como vinho e cerveja (KOWALCZYK et al*.,* 2016).

Assim como para os açúcares, o transporte é uma etapa limitante para a utilização de citrato (MAYO et al., 2010) que pode ser usado como fonte de carbono e/ou energia na presença ou ausência de oxigênio para fornecer intermediários para a formação de biomassa (LAËTITIA *et al*., 2014). Este transporte é realizado por uma variedade de permeases associadas à membrana. Entre os transportadores de citrato estão, por exemplo, o CitS, que é um simporter, que usa um gradiente de Na^{2+} para transportar citrato, o CitP que é responsável pelo antiporte de H-citrato^{2-} e lactato^{1-} gerando um potencial de membrana (MAYO et al., 2010) e CitM e CitH, pertencentes à família de simportadores citrato-metal (CitMHS) (KORITHOSKI et al., 2005; BLANCATO et al., 2006). Uma vez dentro da célula, o citrato pode ser convertido em acetato e oxaloacetato em uma reação catalisada pelo complexo enzimático citrato liase (CL) e, em seguida, o oxaloacetato é descarboxilado pela oxaloacetato descarboxilase (OAD), gerando piruvato, acetado e CO_2 (MAGNI et al., 1999; KOWALCZYK et al*.,* 2016).

As bactérias que metabolizam o citrato parecem ser favorecidas, em particular quando o ambiente se torna severo. Este é o caso de produtos alimentícios fermentados, onde a acidificação diminui o pH em níveis sub-letais (LAËTITIA et al., 2014). A utilização de citrato tem um efeito protetor contra o estresse ácido, excretando o ácido lático produzido à medida que importa citrato utilizando um antiportador CitP, por exemplo (KOWALCZYK et al*.,* 2016), o que poderia fornecer às BALs fermentadoras de citrato um mecanismo de resistência aos efeitos inibitórios do lactato acumulado no meio (MAGNI et al., 1999). Além disso, o metabolismo do citrato contribui para a produção de compostos tóxicos, ácidos orgânicos e diacetil, que apresentam atividades contra muitas bactérias (LAËTITIA et al., 2014; KOWALCZYK et al*.,* 2016). Em um ecossistema microbiano, o metabolismo do citrato oferece ainda, vantagens seletivas para os microrganismos citrato-positivos, podendo ainda gerar um mol extra de ATP por mole de citrato (LAËTITIA et al., 2014; KOWALCZYK et al*.,* 2016) e o seu co-metabolismo permite que as BALs usem outra fonte de carbono para seu crescimento (KOWALCZYK et al*.,* 2016). O benefício energético desse metabolismo permite que as bactérias suportem melhor e, até mesmo, dominem o ambiente na qual estão inseridas (LAËTITIA et al., 2014).

2.3.4. O gênero Lactobacillus

O gênero *Lactobacillus* é um grupo heterogêneo de bactérias ácido láticas (BAL), composto por bactérias com diferentes propriedades fenotípicas, bioquímicas e fisiológicas (AXELSSON, 2004). As principais diferenças, são relacionadas a ter ou não a capacidade de utilizar os carboidratos e de sintetizar os aminoácidos, a depender do nicho onde estes microrganismos são encontrados. (O'CALLAGHAN; O'TOOLE, 2011). São, geralmente, as espécies de bactérias láticas mais ácido-tolerantes e apresentam morfologia bacilar (HAMMES; VOGEL, 1995; HAMMES; HERTEL, 2006; SLOVER; DANZIGER, 2008.), mas a morfologia da coloração de Gram pode variar, incluindo hastes curtas e roliças, hastes longas e delgadas, em correntes ou paliçadas, e sua morfologia colonial pode variar entre colônias pequenas a médias. Todas essa variáveis não permitem que sejam facilmente diferenciadas, pois tornam a identificação fenotípica geralmente não confiável, de forma que frequentemente requerem identificação molecular (genes 16S rRNA). Desta forma, é considerado um grupo taxonomicamente complexo, sendo composto por mais de 170 espécies. Esta complexidade taxonômica, juntamente com a escassez de dados faz com que seja difícil generalizar sobre o gênero (GOLDSTEIN et al., 2015). A heterogeneidade do grupo é também refletida na variação dos valores de GC em seu DNA. Espécies do gênero apresentam variações aproximadamente entre 32 e 55mol%, o que representa cerca de duas vezes mais do que o aceitável normalmente, para um único gênero (SCHLEIFEr, 1983).

Estas bactérias são fastidiosas, apresentando necessidades nutricionais complexas, por vezes muito exigentes para os carboidratos, aminoácidos, peptídeos, ésteres de ácidos graxos, sais derivados de ácidos nucleicos vitaminas e minerais (HAMMES; VOGEL, 1995; HAMMES; HERTEL, 2006; KÖNIG; FRÖHLICH, 2009; HOLZAPFEL; WOOD, 2014). Ocasionalmente são redutoras de nitrato, normalmente não têm motilidade (HAMMES; VOGEL, 1995; HAMMES; HERTEL, 2006) e crescem a uma temperatura e pH ótimos entre 30 e 40ºC, e 5.5 e 6.2, respectivamente (HAMMES; HERTEL, 2009; SALVETTi et al., 2012).

Amplamente distribuídos na natureza, os *Lactobacillus* são encontrados onde substratos ricos em carboidratos estão disponíveis (HAMMES; VOGEL, 1995; HAMMES; HERTEL, 2006), podendo ser também encontrados em diversos produtos alimentícios, principalmente em produtos de origem láctea (AXELSSON, 2004, SALVETTI et al., 2012). Geralmente são considerados como “Bactérias seguras” (SALVETTi et al., 2012), e compõem a microbiota entérica de humanos e outros mamíferos (SEKIROV et al., 2010), sendo um grupo subdominante da microbiota intestinal humana. Sua habilidade de resistência a ácidos, faz com que sejam capazes de sobreviver à ingestão e representam os tipos de microrganismos cujos quais sistema imunológico dos mamíferos aprenderam a não reagir (O’CALLAGHAN; O’TOOLE, 2011). Isto fez com que os *Lactobacillus* ocupassem uma posição importante frente à saúde humana, de forma que algumas espécies, como *Lactobacillus rhamnosus, L. acidophilus, L. casei, L. reuteri e L. fermentum*, fossem utilizadas como probióticos, por exemplo (REID, 1999). Sua capacidade de adaptar às situações adversas, recorrentemente encontradas no ambiente industrial, juntamente com a produção de ácidos orgânicos resultantes de seu metabolismo, fazem com que o gênero *Lactobacillus* seja uma grande preocupação para destilarias e usinas de etanol de combustível (NARENDRANATH, 2003), uma vez que promovem sérias consequências econômicas para os produtores, pois além de desviarem o carbono da formação do etanol prejudicam o desempenho fermentativo da levedura (BASSO et al., 2014)

Na presença de oxigênio ou de outros oxidantes, quantidades elevadas de acetato podem ser produzidas em detrimento ao lactato ou etanol. Assim, variações nos produtos metabólicos finais podem ocorrer. Vários compostos (por exemplo, citrato, malato, tartarato, quinolato, nitrato, nitrito, etc) podem ser metabolizados, e utilizados como fonte de energia ou aceptores de elétrons (HAMMES; VOGEL, 1995). A presença de membros do gênero em uma gama tão diversa de nichos levou à evolução de uma série de diferentes agrupamentos de lactobacilos, cada um adaptado ao nicho ambiental do qual se originou (O’CALLAGHAN; O’TOOLE, 2011). Originalmente, as espécies deste gênero eram agrupadas com base na temperatura de crescimento e habilidade de fermentar hexoses (CARR et al., 2002). Há muitos anos a base fisiológica para a divisão é, geralmente, a presença ou ausência das enzimas do metabolismo de açúcar, dividindo, portanto, o gênero em três grupos distintos, de acordo com o modo como realizam a degradação dos carboidratos (KANDLER, 1983; KANDLER; WEISS, 1986; HAMMES; VOGEL, 1995; SUN et al., 2014): Homofermentadores Obrigatórios (OHO), que só fermentam hexoses à ácido lático, quase que exclusivamente (cerca de 85%, no mínimo), pela via EMP, na qual 1 mol de hexose leva a formação de 2 moles de ácido lático e 2 moles de ATP; Heterofermentadores Facultativos (FHE), que assim como o grupo OHO, fermentam hexoses a ácido lático pela via EMP, mas em condições extremas de glicose limitante, tem habilidade de degradar gluconato e pentoses através da via Fosfocetolase (PK), resultando na produção de ácido lático, etanol e/ou ácido acético e CO_2; e os Heterofermentadores

Obrigatórios (OHE), que fermentam hexoses e pentoses exclusivamente pela via Fosfogluconato, produzindo ácido lático, etanol ou ácido acético e CO_2. Apesar de ainda muito utilizada, essa classificação necessita de atualização, uma vez que não contempla espécies homefermentadora de pentoses, como a *Lactobacillus vini,* única espécie descrita na literatura que apresenta esse metabolismo, e que utiliza a via Pentose Fosfato na assimilação das pentoses, em detrimento da via Fosfocetolase (Figura 2) (BARRE, 1978; RODAS et al*.,* 2006).

2.3.5. Lactobacillus vini

Diferentes estudos realizados até a década de 70, identificaram as espécies de bactérias láticas presentes na produção de vinho, na França, estabelecendo assim a população que compunha este ambiente. O fato de, até então, não haverem sido descritos nesta população indivíduos que crescessem em altas temperaturas, levou P. Barre a desenvolver seu estudo, onde foram isolados *Lactobacillus* do mosto de vinho, que cresciam a temperaturas superiores a 40°C (BARRE, 1978). Dentre as cepas isoladas, estava uma que foi denominada Mont 4. Este isolado foi depositado primeiramente no Deustche Sammlung von Micro-organismem und Zellkulturen GmbH (DSMZ) como *Lactobacillus sp.* DSM 20605 e, posteriormente, na Spanish Type Culture Collection (CECT) da DSMZ como *Lactobacillus sp*. CECT 5924 (RODAS et al., 2006). Esta cepa apresentava características, como temperatura de crescimento na faixa de 45°C e metabolismo homofermentativo, que a aproximava do então subgênero Thermobacterium, mas sua capacidade de fermentar arabinose e ribose não permitia esta classificação. *Lactobacillus* com metabolismo homofermentativo para pentoses eram desconhecidos até então, o que levou à conclusão de que deveria se tratar de uma nova espécie (BARRE, 1978). Muitos anos depois, com a utilização de técnicas moleculares, a hipótese de se tratar de uma espécie ainda não descrita foi reforçada (CHENOLL, et al., 2003). Posteriormente, um estudo que analisou a sequencia do gene 16S rRNA da população de lactobacilos em vinícolas da Espanha encontrou um grupo de isolados que apresentavam 100% de similaridade com a cepa Mont 4 (RODAS et al., 2005), que foi então descrita como a nova espécie *Lactobacillus vini* em 2006 (RODAS et al., 2006)

Lactobacillu vini (Figura 5) é uma bactéria positiva para o teste de Gram, catalase negativa, não esporulante e que apresenta o padrão de metabolismo homofermentativo, mesmo na fermentação de pentoses, produzindo exclusivamente o lactato como produto final da fermentação de carboidratos (BARRE, 1978; RODAS et al., 2006). É anaeróbia facultativa, e entre outros açúcares, fermenta celobiose, maltose, trealose, sacarose, D-glicose, D-frutose, D-manose e L-Arabinose, mas não fermenta D-arabinose, assim como não fermenta glicerol, D-xilose, L-xilose, manitol, lactose, xilitol, D-arabitol ou L-arabitol (RODAS et al., 2006). O genoma de *L. vini*, tanto da linhagem tipo DMS 20605T descrita por Rodas et al. (2006) como da linhagem industrial JP7.8.9 isolada da fermentação alcoólica, foi sequenciado e contém, em média, 2,25 Mb com conteúdo de GC na faixa de 37,7%, que abriga cerca de 1950 genes (LUCENA et al., 2012). As anotações revelaram a presença de genes que tem grande relevância para uso industrial, como no caso de hidrolases e outras enzimas do metabolismo de carboidratos.

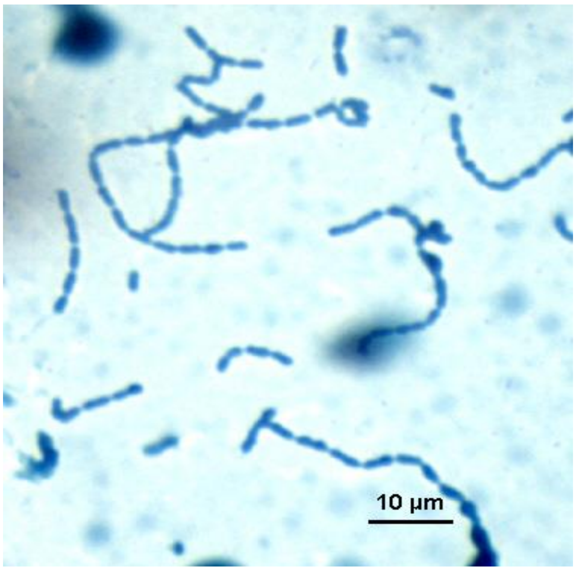

Figura 5. Morfologia das células de *Lactobacillus vini,* linhagem industrial JP7.8.9 depois de coloração de Gram (Fonte: A autora).

Desde sua descrição*, L. vini* vem sendo encontrada em alguns processos industriais pelo mundo, estando sempre relacionada ao processo de fermentação alcoólica. Primeiro na produção de vinho, na França (BARRE, 1978) e na Espanha (RODAS et al., 2005) e, posteriormente, foi detectada na Suécia durante o processo de produção de etanol (PASSOTH et al., 2007). No Brasil, foi isolada a partir do mosto fermentado, de destilarias de etanol do nordeste, se mantendo em maior quantidade com relação a outras BALs e aumentando sua população durante toda a safra 2007/2008 (LUCENA et al., 2010). Estudos posteriores indicaram que influencia da presença dessa bactéria no rendimento da fermentação alcoólica em caldo de cana-de-açúcar sobre o consorcio entre *S. cerevisiae* (linhagem industrial JP1) e *D. bruxellensis* (linhagem industrial GDB 248), não exerce nenhum efeito negativo sobre as células de levedura (DE SOUZA et al., 2012). Dados na literatura sugerem que o sucesso adaptativo deste gênero se deve a sua tolerância às condições do ambiente industrial de fermentação alcóolica, tais como a alta concentração de etanol, à condição anaeróbica, ao baixo pH e a presença de antibióticos, por exemplo (LUCENA et al., 2010; BECKNER et al., 2011; MENDONÇA et al., 2016; MENDONÇA et al., 2019). Apesar destes estudos, ainda pouco se sabe a respeito de sua fisiologia e seu metabolismo, o que permitiria compreender a ecologia deste microrganismo no processo industrial (as características de adaptabilidade e sua relação com os outros componentes da microbiota do processo), bem como seu potencial na produção de compostos de interesse biotecnológico (tais como a produção de ácidos orgânicos e de compostos aromáticos, ou mesmo na produção de biocombustíveis).

3 MÉTODO

3.1 Microrganismos e condições de crescimento

A linhagens utilizadas neste trabalho foram a linhagem industrial *Lactobacillus vini* JP7.8.9, isolada da destilaria de etanol Japungú, Santa Rita, PB (LUCENA et al., 2010), e as linhagens mutantes *relA*$^-$ e *prcR*$^-$ (MENDONÇA, 2018). Todas as linhagens foram cultivadas a 37 °C, em condições estáticas, em meio MRS caldo (Merck e Difco), ou meio sólido (contendo 2% de ágar) em jarra de anaerobiose com gerador de anaerobiose Anaerocult A® (Mikrobiologie). Para os cultivos das linhagens mutantes, o antibiótico eritromicina (Sigma Aldrich ®) foi adicionado ao meio, na concentração final de 5 µg/mL (MENDONÇA, 2018). Todas linhagens foram mantidas em MRS caldo 15% de glicerol a -80 °C. Caldo e melaço da cana-de-açúcar, fornecidos pelas destilarias de Japungu e Miriri (Santa Rita, PB), foram utilizados como substratos industriais. A suplementação desses substratos incluiu a adição de componentes individuais do composto MRS: peptona (10 g L^{-1}), extrato de levedura (5 g L^{-1}), citrato de amônio (2 g L^{-1}) ou uma mistura de vitaminas (D -biotina 0,05 g L^{-1}, ácido pantotênico 1 g L^{-1}, ácido nicotínico 1 g L^{-1}, mioinositol 25 g L^{-1}, cloridrato de tiamina 1 g L^{-1}, cloridrato pirodoxal 1 g L^{-1} e p-aminobenzóico ácido 0,2 g L^{-1}). Os meios de cultura industrias utilizados foram previamente esterilizado por filtração, utilizando filtros de membrana de 0,22 µm. Os meios MRS comerciais, caldo ou sólido, foram esterilizado por autoclavação (a 121 °C por 15 minutos). Alternativamente, o extrato de levedura feito pela lise da levedura industrial Saccharomyces cerevisiae JP1, foi usado para suplementar o meio industrial e simular as condições industriais. As células de levedura foram cultivadas em meio YPD (extrato de levedura 10 g L^{-1}, peptona 20 g L^{-1}, dextrose 20 g L^{-1}) para este procedimento, até a fase estacionária e coletadas por centrifugação em tubos cônicos de 50 mL em 5000 rpm por cinco minutos em temperatura ambiente. A biomassa úmida foi lisada pela adição de nitrogênio líquido e grânulos de vidro (475-600 µm de diâmetro), seguido por agitação em vórtice. O lisado foi filtrado em uma membrana de 0,22 µm (Durapore PVDF. Merck Co., Cat nº P1438) e misturado com uma pequena quantidade do meio industrial. Um equivalente de 5g do lisado celular foi adicionado a cada litro de substrato para simular a presença de conteúdo de células de levedura mortas no ambiente industrial.

3.2 Ensaios de crescimento com caldo de cana e melaço

Células de *L. vini* foram coletadas de uma colônia de placas MRS foram cultivadas em caldo MRS por 16 h a 37 °C sem agitação. As células das culturas foram utilizadas para inocular o caldo MRS (como meio de referência) e para o meio industrial de caldo de cana ou melaço, com ou sem adição dos suplementos, inicialmente a uma concentração inicial de 0,1 $D.O_{600nm}$ no volume total de 1 ml. O caldo de cana e o melaço foram centrifugados a 5.000 rpm por 15 minutos para remoção dos sólidos suspensos, e ambos foram diluídos em água destilada para 100 g L^{-1} de açúcar total. Os cultivos de *Lactobacillus vini* foram realizados em FlowerPlates selados em filme permeável com equipamento de microfermentação BiolectorNA® (m2p-labs, Alemanha) a 32 ° C e umidade constante de 85% e velocidade de 800 rpm. Os experimentos de crescimento foram realizados em anaerobiose com injeção de nitrogênio ultrapuro a 0,5 vvm. As leituras do laser de uma faixa de sólidos suspensos foram realizadas a cada 20 minutos e os valores de espalhamento da luz foram transformados em densidade óptica a partir do fator de calibração definido em laboratório para a suspensão de células de *L. vini*. Nenhuma variação de pH do meio de cultura foi observada.

3.3 Análise da assimilação de açúcar e concentração de glicose

O meio MRS foi preparado in-house a partir de seus componentes listados pelo fabricante (Difco) para testar variações na concentração de glicose e na fonte de carbono. Quatro diferentes fontes de carbono foram analisadas: glicose, frutose, sacarose e maltose. A concentração de todas as fontes de carbono foi de 20 g L^{-1}. A influência da concentração de açúcar no crescimento bacteriano foi testada usando glicose a 20, 40, 60, 80, 100 e 120 g L^{-1}. As células pré-cultivadas em meio MRS foram usadas para esses ensaios de crescimento para inocular o meio específico de MRS na concentração inicial de 0,1 $D.O_{600nm}$ em microplacas estéreis de 96 poços até um volume final de 150 µL e cultivadas em aparelho de microincubação Synergy HT (BioTek, Swissland) a 37 ° C, sob alta agitação. As leituras de absorbância foram feitas a cada vinte minutos. Nenhuma variação de pH do meio de cultura foi observada.

3.4 Ensaios de crescimento variando concentração de citrato

O meio MRS foi reproduzido em laboratório a partir de seus componentes com concentrações variáveis de citrato na forma de sal de amônio. A condição de referência foi o meio MRS contendo concentração de citrato presente no MRS comercial (8,8 mM). Em um meio sem citrato, ele foi substituído por sulfato (8,8 mM) como sal de amônio. O efeito combinado de glicose e citrato foi testado pela mistura de ambos os nutrientes a 2, 20 ou 200 mM cada. O caldo de cana e o melaço foram suplementados com citrato a 8,8 mM. Durante os cultivos, as células foram pré-cultivadas em meio comercial MRS e usadas para inocular meio específico de MRS em concentração inicial de 0,1 $D.O_{600nm}$ em um volume total de 1 ml em FlowerPlates. As placas foram incubadas em microfermentador BiolectorNA® conforme descrito anteriormente. A temperatura foi alterada para 37 ° C para um crescimento ideal. Nenhuma variação de pH do meio de cultura foi observada.

3.5 Ensaios de crescimento e fermentação com glicose, citrato e piruvato

Quatro condições foram testadas para MRS preparado em laboratório: (i) com glicose (20 g L-1) e citrato de amônio (citrato 8,8 mM); (ii) com glicose (20 g L-1) mas sem citrato de amônio; (iii) sem glicose e com citrato de amônio (88 mM); (iv) com glicose (20 g L-1) e piruvato de sódio (piruvato 8,8 mM). Conforme descrito acima, as células pré-cultivadas foram ressuspensas e inoculadas no meio específico de MRS na concentração inicial de 0,1 $D.O_{600nm}$ em um volume de 45 ml em frascos a 37 ° C em condições estáticas. As leituras de absorbância foram realizadas em intervalos de 20, 24 e 48h, sendo que às 24 e 48h as amostras foram centrifugadas e o sobrenadante foi reservado para análise do consumo de açúcar e produção de metabólitos de acordo com os padrões do protocolo descritos a seguir. Todas as fermentações foram realizadas em triplicado, sendo os resultados obtidos representados como média simples (± desvio padrão). Nenhuma variação de pH do meio de cultura foi observada.

3.6 Análise de assimilação dos açúcares e crescimento celular em diferentes fontes de carbono

Para os ensaios de variação de fonte de carbono, o meio MRS foi reproduzido em laboratório a partir de seus componentes, segundo o fabricante (Merck), alterando-se os tipos de açúcar a serem testados (glicose, frutose, sacarose, celobiose e lactose) na concentração de 20 gL^{-1}. As células foram pré-cultivadas em meio MRS Merck e utilizadas para inocular os meios MRS-específicos para concentração inicial de 0,1 DO_{600nm}. Os cultivos foram conduzidos em sistema de batelada simples sem agitação durante 48 horas à 37ºC. As amostras coletadas foram utilizadas para leitura da absorbância em espectrofotômetro a 600nm. Em seguida foram centrifugadas e o sobrenadante reservado para análise de consumo dos açúcares e produção de metabólitos conforme protocolo descrito abaixo. Todas os cultivos foram realizados em duplicatas biológicas e triplicatas técnicas, e os resultados obtidos foram representados como médias simples (± desvio padrão).

3.7 Crescimento celular em duas fontes de carbono

As células de bactérias pré-cultivadas em meio MRS foram coletadas e re-suspensas para concentração celular inicial de 0,1 DO_{600nm} nos diferentes meios MRS reconstituídos, contendo agora, como fontes de carbono: (A) glicose 10 gL^{-1} + sacarose 10 gL^{-1} e (B) glicose 10 gL^{-1} + celobiose 10 gL^{-1}, totalizando 20 gL^{-1} de açúcares totais em cada meio (A e B). Os cultivos foram conduzidos em sistema de batelada simples sem agitação durante 24 horas à 37ºC. As amostras foram coletadas e analisadas conforme descrito acima.

3.8 Indução do crescimento em meio contendo lactose

O meio MRS foi reproduzido conforme fabricante, e a lactose foi utilizada como fonte de carbono, em substituição à glicose. As células bacterianas foram cultivadas em meio MRS comercial, coletadas e re-suspensas para concentração celular inicial de 0,1 DO_{600nm} em meio MRS reconstituído, contendo 20 gL^{-1} de lactose, ou 20 gL^{-1} de lactose com adição de 0,2 gL^{-1} de glicose, ou 20 gL^{-1} de lactose 0,2 gL^{-1} de celobiose. Os cultivos foram realizados sem agitação durante 48 horas à 37ºC. As amostras coletadas e analisadas conforme descrito acima.

3.9 Análise metabólica

As amostras coletadas ao longo das incubações foram centrifugadas para coleta do sobrenadante, que foi filtrado em membrana 0,22mm, para determinação da concentração do açúcar residual e dos metabólitos produzidos, por cromatografia líquida de alta pressão (HPLC) em equipamento Agilent Technologies 1200 Series com a coluna modelo HPX87H (BioRad) em fase móvel de ácido sulfúrico a 5 mM, a 45ºC. A identificação dos metabólitos foi feita por comparação dos tempos de retenção com soluções-padrão e a concentração de cada metabólito foi determinada por integração de picos do cromatograma e relação da área de cada pico com o fator de calibração de cada solução-padrão, através da metodologia de ZHAO et *al*., (2009) adaptada por DE SOUZA et al*.,* 2012.

4 RESULTADOS

4.1 ARTIGO 1 Nutritional requirements for *Lactobacillus vini* growth in sugarcane derivative substrate of ethanol fermentation.

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Abstract

Lactobacillus vini is a bacterial contaminant found in industrial environments of winemaking and fuel-ethanol fermentation. However, there has been no standard analysis of its physiology that can pinpoint its adaptive traits to these kinds of environments. In view of this lack of information, the aim of this study is to determine the nutritional factors that lead to the growth of *L. vini* in the industrial plants of fuel-ethanol. First of all, the limited growth of this bacterium was studied in the industrial substrate, which was improved by the nutritional supplementation of amino acids, and its homofermentative status was confirmed. Metabolite analysis showed that citrate is a growth factor of paramount importance for this bacterium in industrial processes through its pyruvate metabolization, and increases ATP production and biomass formation. Furthermore, the acetate uptake, either from the medium or generated from citrate metabolism, was assimilated for biomass production. Hence, a metabolic model was designed to describe the role of citrate and acetate in the growth of *L. vini* that could be tested on other lactobacilli.

Key words: ATP yield; carbon balance; citrate; Lactobacilli nutrition; molasses; sugarcane; sugar metabolism.

Introduction

The diversity of lactic acid bacteria (LAB) in fermentation processes has been described in different environments, such as (i) the cornstarch-based ethanol production in the United States, (ii) the fermentation of barley wort in craft beer production and (iii) the fermentation of grape wort in wine production, as well as (iv) the ethanol production industry in Vietnam (Skinner and Leathers 2004; Rodas, Ferrer and Pardo 2005; Schell *et al.* 2007; Thanh, Mai and Tuan 2008; Garofalo *et al.* 2015; Lonvaud-Funel 2015; Duar *et al.* 2017). Fuel ethanol distilleries in Brazil use either sugarcane juice or sugarcane molasses, or sometimes a mixture of the two, as a substrate for fermentation. A number of yeasts, *Saccharomyces cerevisiae* and non-*S. cerevisiae* (Basílio *et al.* 2008), and homo- or heterofermentative bacteria (Lucena *et al.* 2010; Basso *et al.* 2014) are present in different cell counts in this ethanol fermentation process. Those most commonly found include *Lactobacillus fermentum*, *L. brevis*, *L. plantarum* and *Weissella paramesenteroides*. However, the *L. vini* species has attracted a good deal of attention on account of its high frequency and abundance among the LAB species in the distilleries of North- East Brazil (Lucena *et al.* 2010).

Although it was only described as a species in 2006, *L. vini* was first found in winemaking in 1978, when it was called the Mont 4 strain (Rodas *et al.* 2006). Later, *L. vini* was also identified as a contaminant in the ethanol production industry in Sweden and Brazil (Passoth, Blomqvist and Schnürer 2007; Lucena *et al.* 2010). These studies discovered that *L. vini* can be harmful to ethanol production by (i) competing for the sugar with the main yeast, *S. cerevisiae*; (ii) producing lactic acid that can be toxic for *S. cerevisiae*; and (iii) through its presence somehow inducing the formation of flocs with *D. bruxellensis* and stimulating the fermentative metabolism of this yeast, while impairing the recycling of the yeast biomass (Passoth, Blomqvist and Schnürer 2007; de Souza *et al.* 2012; Tiukova, Eberhard and Passoth 2014).

The *L. vini* strain (described above) was classified as facultative anaerobic and homofermentative for hexose and pentose, since it only produces lactate as an end product (Rodas *et al.* 2006). Moreover, it can metabolize cellobiose, maltose, trehalose, sucrose, D-glucose, D-fructose, D-mannose and L-arabinose, but is not able to make use of glycerol, D-xylose, L-xylose, mannitol and lactose (Rodas *et al.* 2006). However, it should be noted that there is a wide metabolic variability among the LABs, especially the Lactobacillus group, even within the same species (Sun *et al.* 2014). This variation can be observed by the number of phosphotransferase systems (PTS) whose variability can be explained by the environmental pressure on substrate activity with a high concentration of different types of carbohydrates (Aleksandrzak-Piekarczyk 2013; Zhang *et al.* 2013). Moreover, LABs can be classified as either heterofermentative or homofermentative, depending on the type of metabolic pathway used to produce lactate, acetate and/or ethanol/CO_2, such as the glycolytic pathway, phosphate-pentose pathway and phosphoketolase pathway (Kandler 1983). Hence, this limited amount of information does not allow a comprehensive analysis to be conducted of the metabolism of *L. vini*, especially with regard to its ability to adapt to industrial environments such as those where sugarcane derivatives are used as substrates. Recent studies have reported the tolerance of *L. vini* to several forms of stress found in the fermentation process, such as acidification by organic and inorganic acids (Mendonça *et al.* 2019). In this regard, we showed that *L. vini* can take advantage of the substrate composition whenever some amino acids are present, which helps to keep the internal pH unaltered under acidic stress (Mendonça *et al.* 2019). Thus, some mechanisms for adapting to these environments have begun to be revealed.

Sugarcane juice is a quite simple substrate in terms of its main components when compared with barley or wheat wort, although its micro-composition can vary. Apart from the energy value of the high sugar content, several organic substances are found in the different sources of the cane, such as vitamins, phosphatides and amino acids (reviewed by Arif *et al.* 2019). This last group of components can be found in up to 2.5% (w/v) of the juice (Arif *et al.* 2019) and might be a key factor for bacterial acid tolerance, as stated above. The mineral composition is caused by the genetic diversity of the cane and soil quality (de Souza *et al.* 2015; Arif *et al.* 2019), and affects its fermentability when in a high concentration (de Souza *et al.* 2015).

The aim of this work is to study the metabolism of *Lactobacillus vini* JP7.8.9, a strain that is isolated from industrial ethanol fermentation (Lucena *et al.* 2010), in order to explain its high adaptability to the fermentative environment, especially in response to the nutritional composition of sugarcane juice. Hence, it was hoped the study would reveal the essential features of the *L. vini* metabolism that have practical repercussions on its behaviour in industrial fermentation processes.

Material and Methods

Bacteria strain and culture media

The strain of LAB used in this work was *Lactobacillus vini* JP7.8.9, which was isolated in the fuel-ethanol distillery of Japungu, located in Paraiba State, Brazil (Lucena *et al.* 2010). MRS broth (Difco Co., USA. Cat. Nº 288130) was used for cell cultivation, henceforth designated as commercial MRS. Solid MRS was prepared by adding bacto-agar to 2% (w/v). Sugarcane juice and molasses (as industrial substrates) were provided by distilleries at Japungu and Miriri (Santa Rita, PB). Supplementation of these substrates included the addition of individual components of the MRS compound: peptone (10 g L^{-1}), yeast extract (5 g L^{-1}), ammonium citrate (2 g L^{-1}) or a mix of vitamins (D-biotin 0.05 g L^{-1}, pantothenic acid 1 g L^{-1}, nicotinic acid 1 g L^{-1}, myoinositol 25 g L^{-1}, thiamine hydrochloride 1 g L^{-1}, pyrodoxal hydrochloride 1 g L^{-1} and p-aminobenzoic acid 0.2 g L^{-1}). All the medium components were purchased from Sigma-Aldrich Co. (St. Louis, USA). All the media liquid used was sterilised by filtration in advance, using 0.22 µm membrane filters. Solid medium was sterilised by autoclavation (at 121ºC for 15 minutes). Alternatively, yeast extract made by the lysis of the industrial yeast *Saccharomyces cerevisiae* JP1, was used to supplement the industrial media and mimic industrial conditions. The yeast cells were cultured in YPD medium (yeast extract 10 g L^{-1}, peptone 20 g L^{-1}, dextrose 20 g L^{-1}) for this procedure, up to the stationary phase and collected by centrifugation in 50 mL conical tubes at 5000 rpm for five minutes at room temperature. The wet biomass was lysed by the addition of liquid nitrogen and glass beads (475-600 µm in diameter), followed by vortex shaking. The lysate was filtered in a membrane of 0.22 µm (Durapore PVDF. Merck Co., Cat nº P1438) and mixed with a small amount of the industrial media. An equivalent of 5g of the cell lysate was added to each litre of substrate to mimic the presence of dead yeast cell contents in the industrial environment.

Growth assay with sugarcane juice and molasses

L. vini cells collected from one colony of MRS plates were cultured in MRS broth for 16 h at 37ºC without shaking. Cells from the cultures were used to inoculate MRS broth (as the reference medium) and to the industrial media of cane juice or molasses, with or without the addition of the supplements, initially to 0.1 absorbance units (A_{600nm}) in the total volume of 1 ml. Sugarcane juice and molasses were centrifuged at 5000 rpm for 15 minutes to remove the suspended solids, and both were diluted with distilled water to 100 g L^{-1} of total sugar. *Lactobacillus vini* cultivations were performed in FlowerPlates sealed in permeable film with

BiolectorNA® microfermentation equipment (m2p-labs, Germany) at 32 °C and at constant humidity of 85% and speed of 800 rpm. Growth experiments were carried out in anaerobiosis with ultrapure nitrogen injection at 0.5 vvm. The laser readings of a range of suspended solids were carried out every 20 minutes and the light scattering values were transformed into optical density from the calibration factor defined in the laboratory for the suspension of *L. vini* cells. No culture medium pH variation was observed.

Analysis of sugar assimilation and glucose concentration

The MRS medium was prepared in-house from its components listed by the manufacturer (Difco) to test variations in glucose concentration and carbon source. Four different carbon sources were analysed: glucose, fructose, sucrose and maltose. The concentration of all the carbon sources was 20 g L^{-1}. The influence of sugar concentration on bacterial growth was tested by using glucose at 20, 40, 60, 80, 100 and 120 g L^{-1}. Cells pre-cultured in MRS medium were used for these growth assays to inoculate the MRS-specific media at initial concentration of 0.1 OD_{600nm} in sterile 96-well microplates to a final volume of 150 µL and grown in Synergy HT microincubator apparatus (BioTek, Swissland) at 37 °C, under high agitation. Absorbance readings were taken every twenty minutes. No culture medium pH variation was observed.

Growth experiments of varying citrate concentration

MRS medium was reproduced in the laboratory from its components with varying concentrations of citrate in the form of ammonium salt. The reference condition was MRS medium containing citrate concentration present in commercial MRS (8.8 mM). In a medium lacking citrate, it was replaced by sulphate (8.8 mM) as ammonium salt. The combined effect of glucose and citrate was tested by mixing both nutrients at 2, 20 or 200 mM each. Sugarcane juice and molasses were supplemented with citrate at 8.8 mM. During the cultivations, the cells were pre-cultured in commercial MRS medium and used to inoculate MRS-specific media at baseline of 0.1 OD_{600nm} in a total volume of 1 ml in FlowerPlates. The plates were incubated in BiolectorNA® microfermentor as previously described. The temperature was changed to 37 °C for optimal growth. No culture medium pH variation was observed.

Growth and fermentation experiment with glucose, citrate and pyruvate

Four conditions were tested for in-house made MRS: (i) with glucose (20 g L^{-1}) and ammonium citrate (8.8 mM citrate); (ii) with glucose (20 g L^{-1}) but without ammonium citrate;

(iii) without glucose and with ammonium citrate (88 mM); (iv) with glucose (20 g L^{-1}) and sodium pyruvate (8.8 mM pyruvate). As described above, pre-cultured cells were re-suspended and inoculated into the MRS-specific media at initial concentration of 0.1 OD_{600nm} in 45 ml volume in flasks at 37 °C under static conditions. Absorbance readings were performed at intervals of 20, 24 and 48h, and at 24 and 48h samples were centrifuged and the supernatant was reserved for analysis of sugar consumption and the production of metabolites according to the protocol standards described below. All the fermentations were performed in triplicate, and the results obtained were represented as a simple mean average (± standard deviation). No culture medium pH variation was observed.

Metabolic analyses

The residual sugar concentration and metabolites produced were determined by high pressure liquid chromatography (HPLC) in Agilent Technologies 1200 Series equipment with the model HPX87H column (BioRad) in mobile phase of sulfuric acid to 5 mM at 0.6 mL min^{-1} and 45 °C. The metabolites were identified by means of a refractive index detector with the aid of a standards solution through the methodology described (Zhao and Bai 2009) with adaptations (de Souza *et al.* 2012).

Results and Discussion

Growth analysis of *Lactobacillus vini* in industrial media

Growth experiments were carried out that used sugarcane juice or molasses as an industrial substrate, and laboratory medium MRS as the reference condition. The results showed a slower growth of *L. vini* strain JP7.8.9 in the industrial substrates than was the case with MRS (Fig. 1). Hence, the composition of industrial substrates lack any of the essential factors that inhibit bacterial growth, despite high counts of its presence in many industrial samples of ethanol fermentation (Lucena *et al.* 2010). In view of this paradox, we investigated the nutritional limitations of the sugarcane juice and molasses that impair bacterial growth and the factors that enable *L. vini* growth in such poor substrates. The first condition examined was the fact that the concentration of sugar in the industrial substrates (120 to 140 g L^{-1}) was higher than in MRS (20 g L^{-1}). The variation in glucose concentration from 20 to 120 g L^{-1} had absolutely no effect on the growth pattern of *L. vini* JP7.8.9 (Fig. 2A) which meant that the excess of sugar in the industrial substrate was not the cause of the low growth of the bacterium. In addition, sugarcane juice and molasses contain sucrose together with their hydrolysis products of glucose and fructose (Arif *et al.* 2019). There was no significant difference in growth when the glucose was replaced with sucrose or fructose, which suggests that the composition of sugars was also not related to the growth deficiency in the industrial media (Fig. 2B). Maltose, an □-disaccharide of glucose mainly found in beer wort, was used as an example of a different industrial substrate. Growth experiments showed that maltose was less efficiently used than the other sugars tested (Fig. 2B), and thus that sugar composition could be a determinant for this deficiency in another industrial setting, but not for sugarcane derivatives.

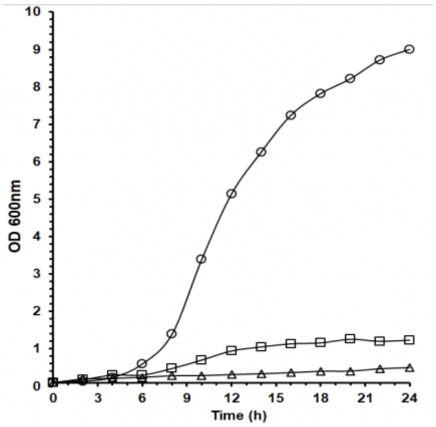


Fig. 1. Growth curve of *Lactobacillus vini* JP7.8.9 in MRS medium containing glucose at 20 g L^{-1} (O) and in industrial media sugarcane juice (□) or molasses (△) containing total sugar at 100 g L^{-1}.

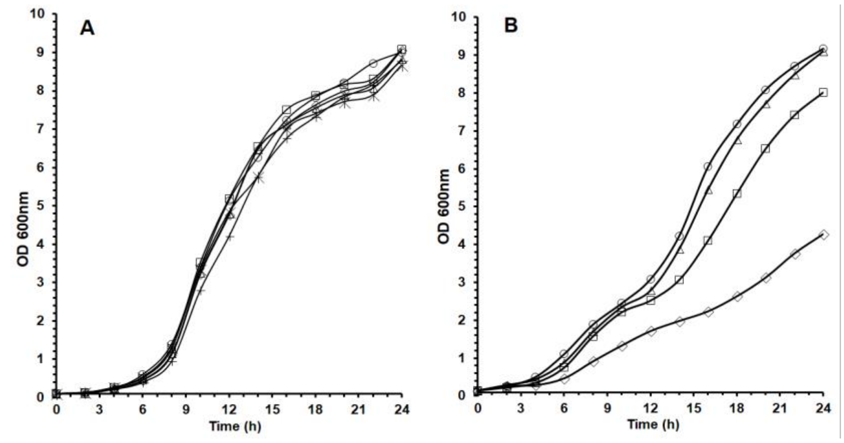


Fig. 2. Effect of the sugar on the growth of *Lactobacillus vini* JP7.8.9 in MRS medium. (**A**) Effect of varying concentrations of glucose at 20 g L^{-1} (O), 40 g L^{-1} (□), 60 g L^{-1} (△), 80 g L^{-1} (◇), 100 g L^{-1} (★) and 120 g L^{-1} (+). (**B**) Effect of different sugars such as glucose (O), fructose (□), sucrose (△) or maltose (◇) at 20 g L^{-1}.

After ruling out the effect of sugar on growth efficiency, we turned our attention to other components of the MRS medium. In this case, industrial substrates were supplemented with peptone, yeast extract and/or a mixture of vitamins. The addition of peptone with or without vitamins or addition of yeast extract alone equally increased bacterial growth in sugarcane juice to the same extent (Fig. 3A) or sugarcane molasses (Fig. 3B), although the final biomass only reached 55% and 40% of that obtained with MRS medium, respectively. This suggested that the fastidious nature of *L. vini*, which is a characteristic of lactobacilli (Duar *et al.* 2017), did not allow its growth in substrates that are low in amino acids, the common nutrients in both supplements. In view of this, the reported presence of *L. vini* in the industrial substrates might be because the bacterial growth was assisted by some sort of supplementation within these compounds.

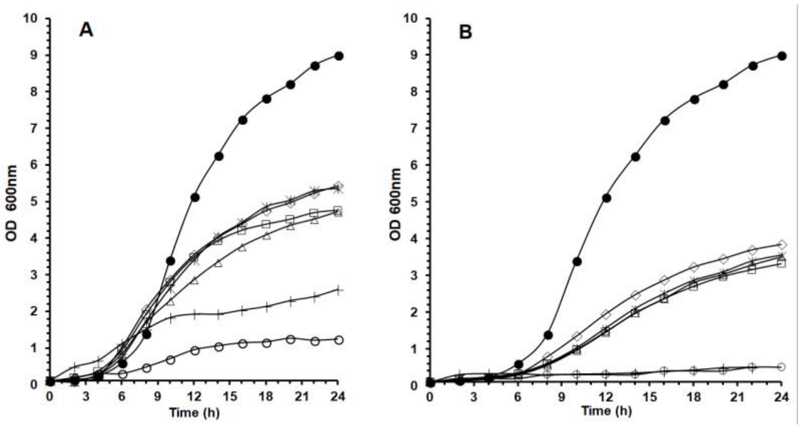


Fig. 3. Effect of supplementation of industrial substrates such as sugarcane juice (○, panel **A**) or sugarcane molasses (○, panel **B**) on the anaerobic growth of *Lactobacillus vini* JP7.8.9 with yeast extract (☐), peptone (△), yeast extract+peptone (◇), peptone+vitamins (★) or yeast lysate (+). MRS medium (●) was used as reference.

One possible source of these kinds of nutrients could be the intracellular contents of dead cells of the microbial population during the fermentation cycles. To test this hypothesis, we supplemented the substrates with a lysate of *S. cerevisiae* industrial strain JP1 in an attempt to mimic the industrial conditions in which the yeast cells are dying. In this case, bacterial growth in lysate-supplemented juice was twice as high as in non-supplemented juice (Fig. 3A), which is evidence of the positive effects of the intracellular content of yeast on bacterial growth. However, this was still at a much lower level than that observed when commercial yeast extract was added (Fig. 3A). It should be noted that this experiment used the estimate of 10% of cell lysis that was calculated for the yeast population in each fermentation cycle (Basso, Basso and Rocha 2011). It resulted in a supplementation of the substrate that was close to 0.1% (w/v) with intracellular content. This concentration is ten times lower than the amount used with commercial yeast extract, which might explain such a difference in growth. Our previous results showed that the count of *L. vini* remained stable when co-cultivated with the yeast *S. cerevisiae* and *D. bruxellensis* in sugarcane, - a condition in which the yeast cells remain intact (de Souza *et al.* 2012). It is nevertheless reported that there was a greater presence of *L. vini* in the fermentation when the industrial substrate was treated with pasteurisation which might lead to the lysis of the microbial autochthonous population of cane juice (de Souza *et al.* 2012). Thus, the data provided here should indeed reflect the industrial conditions, where the bacterial population growth is limited and does not surpass the yeast population.

However, this supplementation did not promote growth in sugarcane molasses (Fig. 3B). This can be explained by the composition of this substrate which contains harmful compounds, such as oxidized molecules produced during the sugar milling, and the high content of the minerals and metals that are concentrated by evaporation during the sugar production. It would require a much higher content of this material released by dead yeasts to allow the growth of *L. vini* in this substrate, as was observed when commercial yeast extract was added to molasses (Fig. 3B). It also explains the absence of *L. vini* in most of the industrial plants that use molasses as a fermentation substrate, which it is not the case for other lactobacilli (Lucena *et al.* 2010).

Influence of citrate on *L. vini* growth patterns

As well as peptone and yeast extract, MRS contains citrate in the form of ammonium salt, which is widely described in the literature as supporting lactobacilli growth (Laëtitia, Pascal and Yann 2014; Kowalczyk *et al.* 2015) and industrial processes (Comasio *et al.* 2019). The role of this six-carbon organic acid in bacterial growth was tested by replacing ammonium citrate (AC) with ammonium sulphate (AS) in MRS composition, while keeping the equimolar quantities of ammonium ion. The absence of citrate extended the lag growth phase (Fig. 4A). After this prolonged non-growth period, the cultures in citrate-lacking MRS started to grow slowly and after 36 h reached the same final biomass of the cultures in citrate-containing MRS (data not shown). This result proved that citrate is important to fasten growth of *L. vini*, but is not indispensable for its long-term growth. Afterwards, the effect of different citrate concentrations in the final biomass was tested. The results showed that at least 2.5 mM of this substrate was necessary to boost the bacterial growth and concentrations above 7.5 mM had a slightly further increment in this parameter (Fig. 4B). Hence, sugarcane juice was supplemented with citrate to the same concentration found in the original composition of MRS (8.8 mM).

Sugarcane juice only supported 15% of the final bacterial growth with regard to MRS (Fig. 4C). The addition of citrate to sugarcane juice doubled the bacterial growth, and reached 30% of the reference condition, while the presence of peptone or yeast extract supported bacterial growth to 50%. A clear additional effect of citrate was observed for substrates supplemented with either peptone or yeast extract, and increased bacterial growth until it reached 90% of the reference condition (Fig. 4C). This means that, as well as the bacterial requirements for amino acids and/or oligopeptides present in peptone or in the yeast extract, citrate is an important growth factor for this bacterium in the industrial substrates.

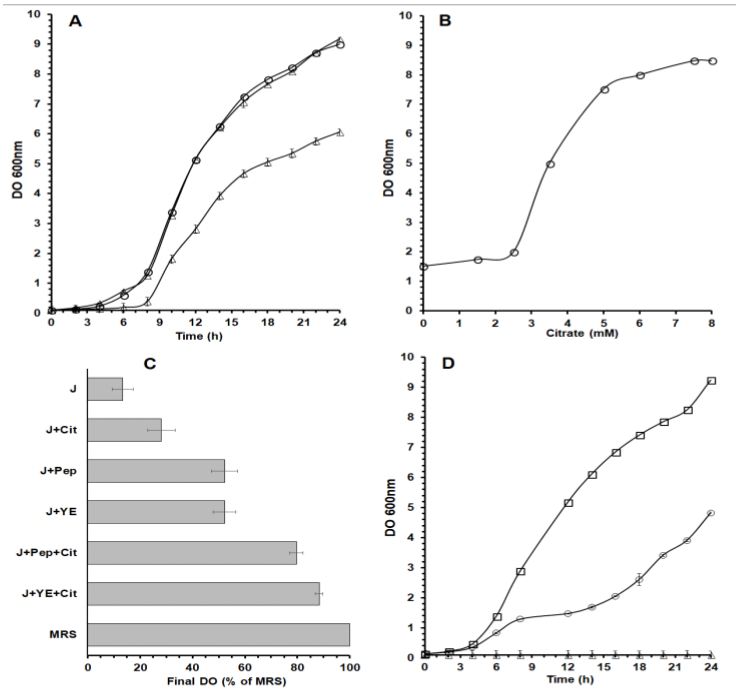


Fig. 4. Influence of citrate on the growth of *Lactobacillus vini* JP7.8.9. (A) commercial MRS (○), MRS medium prepared in-house with ammonium citrate (□) and MRS prepared in-house with ammonium sulphate (△). (**B**) Final bacterial biomass achieved in MRS medium prepared in-house containing varying amounts of ammonium citrate in different concentrations. (**C**) Final bacterial biomass in sugarcane juice (J) with or without supplementation with yeast extract (YE), peptone (Pep), citrate (Cit) relative to complete MRS medium (100%). (**D**) Effect of varied concentrations of ammonium citrate at 2 mM (○), 20 mM (□) or 200 mM (△) on bacterial growth in MRS medium containing glucose at 20 mM. N.B. All citrate concentrations mentioned referred to the citrate anion, not to the salt form.

Finally, an assessment was made to determine whether citrate can also be used as a sole carbon source or in glucose-limited condition. No growth was detected at the initial glucose concentration of 2 mM and a slight growth was observed at 20 mM glucose, irrespective of the presence of citrate (data not shown). The results showed that citrate is not being used by the bacterial cells as the sole carbon source. Additionally, no bacterial growth was observed at 200 mM citrate, whatever glucose concentration was present in the medium. This concentration is close to that used as a food preservative owing to its toxic effects on most of the bacterial cells, which also seems to be the case for *L. vini*. Among the combinations tested, optimal bacterial growth was achieved at 200 mM glucose and 20 mM citrate (Fig. 4D). It should be further evaluated if the increase in growth obtained when citrate was at 20 mM, offsets the increase of the cost of this nutrient in concentrations above 7.5 mM. Altogether, these results found that citrate is mainly used as a supplementary nutritional source in a medium containing enough glucose to provide the carbon needed for *L. vini* growth. There is an abundance of citrate in nature and it is regarded as an important energy source for the growth of different bacteria found in food matrices (Laëtitia, Pascal and Yann 2014).

The effect of citrate on the *L. vini* metabolism

It has been theoretically conjectured that lactobacilli metabolize citrate (6C) through its initial breakdown to acetate (2C) and oxaloacetate (4C) by means of citrate lyase, and that the oxaloacetate is further decarboxylated to pyruvate (3C) (Hugenholtz 1993; Kowalczyk *et al.* 2015). Hence, pyruvate could become the product of citrate metabolization that is related to energy production. Since this route would be redox neutral, pyruvate cannot be reduced to lactate and must thus be deviated to the production of acetyl-CoA. As well as this, acetyl-CoA is further converted to acetyl-P using inorganic phosphate. This energy-rich molecule is the substrate for ATP production that results in acetate (Laëtitia, Pascal and Yann 2014), which can be either a) expelled to the medium, b) converted to acetoin or diacetyl by the bacterial cells (Hugenholtz 1993), or c) further assimilated into the biomass. In view of this possibility, we replaced citrate with its metabolization product (pyruvate) at a concentration of 8.8 mM in MRS. The results showed that both citrate and pyruvate resulted in the same final biomass at 24 h of cultivation (Table 1), which proves that pyruvate indeed metabolically replaces citrate. This finding provides experimental evidence that corroborates the use of metabolic models proposed for other lactobacilli (Hickey, Hillier and Jago 1983; Quintans *et al.* 2008). Hence, it can be concluded that pyruvate is ultimately responsible for the increment in the growth of *L. vini* in the industrial substrates supplemented with citrate (Fig. 4).

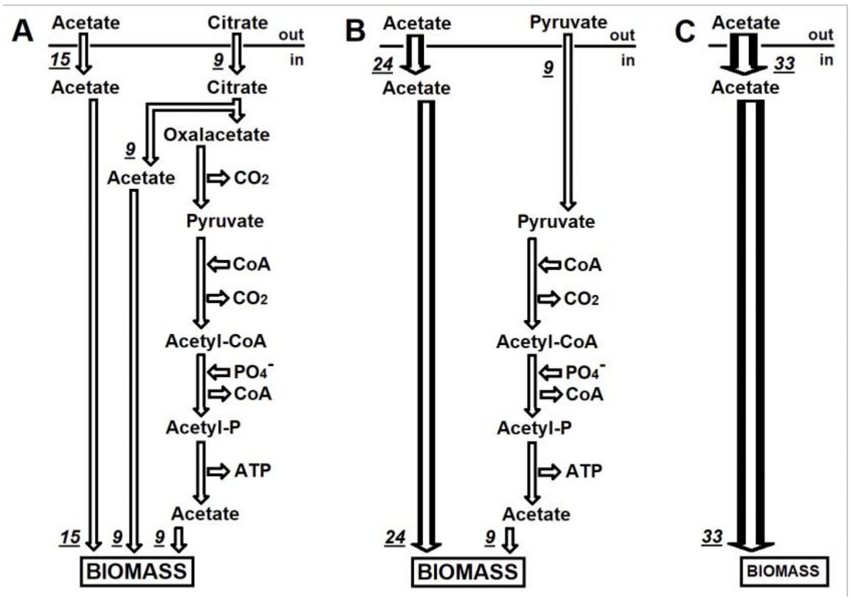
Final concentrations of metabolic products were measured from cultures of *L. vini* in different medium compositions after 48 h of cultivation (Table 1). Regardless of the presence of citrate or pyruvate, the cells completely consumed the glucose and produced lactate at about 70% of the maximum theoretical efficiency as a single fermentation product (Table 1) and as a result, confirmed the homofermentative status given to *L. vini* (Rodas *et al.* 2006). This high conversion efficiency shows the potential of *L. vini* for the industrial production of lactate, an important bioproduct with many applications for the synthesis of biodegradable polymers (Alves de Oliveira *et al.* 2018). When citrate or pyruvate were added to the medium, the final biomass increased by about 30% relative to the medium without citrate (Table 1). As a result, the carbon from citrate or pyruvate is assimilated for cell formation and is not dissimilated to a fermentation product such as CO_2, acetate of ethanol. It also led to the conclusion that the acetate that is first produced by the breakdown of citrate and ultimately formed from pyruvate, is assimilated into the biomass.

Table 1. Final concentrations of metabolites and biomass from cultivation of *Lactobacillus vini* JP7.8.9 for 48 h in MRS prepared in-house with different formulations[a].

Parameter	Glu (112 mM) Ace (61 mM)	Glu (112 mM) Ace (61 mM) Cit (8.8 mM)	Glu (112 mM) Ace (61 mM) Pyr (8.8 mM)
Glucose (mM)[b]	-112	-112	-112
Acetate (mM)[b]	-31 (±0.1)	-15.3 (±0.32)	-22.7 (±0.21)
Citrate (mM)[b]	n.a.[e]	-8.8	n.a.
Pyruvate (mM)[b]	n.a.	n.a.	-8.8
Biomass (OD_{600nm})[c]	6.06 (±0.047)	9.09 (±0.087)	9.08 (±0.086)
Lactate (mM)[c]	157.1 (±11.6)	165.8 (±13.6)	170.3 (±16.1)
Lactate yield (g g^{-1})[d]	0.70	0.74	0.76

[a]Glu: glucose; Ace: acetate; Cit: citrate; Pyr: pyruvate. [b]metabolite consumed. [c]metabolite produced. [d]lactate produced/glucose consumed. [e]not applicable.

As well as citrate, acetate is also present in MRS at an initial concentration of 61 mmol/L and our findings showed that this metabolite was differentially consumed by the bacterial cells in the different media: 33 mmol consumed in MRS+glucose, 24 mmol consumed in MRS+glucose+pyruvate and 15 mM consumed in MRS+glucose+citrate (Table 1). Hence, less external acetate was required when citrate was co-consumed with glucose. In the case of MRS+glucose+citrate, the bacterial cells consumed 9 mmol less acetate from the medium than in MRS+glucose+pyruvate. This is in accordance with the calculation that the breakdown of 8.8 mmol citrate present in MRS first releases 8.8 mmol acetate and then 8.8 mmol of oxaloacetate, which ultimately releases a further 8.8 mmol acetate from pyruvate. This results in 17.6 mmol acetate available for biomass formation and was very close to the experimental difference of 16 mmol between the MRS medium with or without citrate (Table 1). If pyruvate was used in the medium, 9 mmol more acetate was experimentally consumed by the cells than the citrate medium (Table 1). This is once again very close to the hypothesis that there is a metabolic deficit of 8.8 mmol acetate when pyruvate is metabolised. From these data, it was possible to devise a metabolic model, which is closely in line with that previously proposed (Laëtitia, Pascal and Yann 2014), and suggests that citrate is metabolised to generate ATP, as well as to ultimately produce acetate that is used together with the external acetate for biomass formation (Fig. 5). Pyruvate is thus able to fulfil the citrate requirement for ATP production, although it imposes the need for more acetate uptake from the medium (Fig. 5). This uptake is even higher in the absence of citrate or extracellular pyruvate (Fig. 5).


A
Acetate
Citrate
out
in
15
9
Acetate
Citrate
Oxalacetate
9
Acetate
CO2
Pyruvate
CoA
CO2
Acetyl-CoA
PO4⁻
CoA
Acetyl-P
ATP
Acetate
15
9
9
BIOMASS
B
Acetate
Pyruvate
out
in
24
9
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Pyruvate
CoA
CO2
Acetyl-CoA
PO4⁻
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Acetyl-P
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Acetate
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33
Acetate
33
BIOMASS

Fig. 5. Proposed metabolic pathways for the growth of *Lactobacillus vini* JP7.8.9 in medium containing acetate and citrate (panel A), acetate and pyruvate (panel B) or solely acetate (panel C). Metabolites in the medium (out) involves cellular uptake (in) and are used for biomass formation. The underlined numbers represent the amount of metabolite assimilated. The width of the arrow related to the assimilation of extracellular acetate represents the amount of this metabolite. The size of the source indicating biomass formation, represents the comparison of the final biomass in each growth condition.

Acetate is the building block for lipid biosynthesis in reactions that use NADPH as an electron donor. This reduced coenzyme is produced in the pentose phosphate (PPP) pathway from glucose. The data in Table 1 showed that 70% of glucose is converted to lactate, while suggesting that 33.6 mmol of glucose (30%) deviated to PPP to generate 67.2 mmol NADPH. This amount is theoretically enough to convert 34 mmol acetate to lipids, which was the exact amount calculated from the consumption of acetate (Table 1). The bacterial cells grow slowly when the biosynthesis of lipid depends entirely on the influx of acetate, in the absence of citrate (Fig. 4A), and takes a longer incubation period to reach the same final biomass obtained in complete MRS. In the light of this, it can be concluded that the rate of growth of *L. vini* JP7.8.9 is limited by the rate of acetate uptake. The stimulating effect of citrate on the growth of lactobacilli was pointed out in the original description of the MRS medium (Man, Rogosa and Sharpe 1960). Since then, no explanation was given for the use of citrate until the recent metabolic model postulated that this nutrient played a role in the growth of lactobacilli (Laëtitia, Pascal and Yann 2014). Hence, this study is the first to provide consistent experimental data that at least explain this nutritional requirement for *L. vini* and assist in elucidating the behaviour of this bacterium in industrial fermentation processes.

Conclusion

This study has shown that the limited growth of *L. vini* in sugarcane derivative substrates is the consequence of a lack of oligopeptides/amino acids and citrate as key nutritional requirements. This limitation can be overcome by the lysis of the part of the microbial population, (mainly yeast cells), that release these supplements in the substrate and allow bacterial cells to grow during the fermentation process. It also consolidates the metabolization of citrate through pyruvate, by providing enough acetate for biomass formation. This information on *L. vini* metabolic features reveals significant aspects of its performance in industrial fermentation processes.

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References

Aleksandrzak-Piekarczyk T. Lactose and β-Glucosides Metabolism and Its Regulation in *Lactococcus lactis*: A Review. In: Kongo, J. Marcelino (University of the Azores P (ed.). *Lactic Acid Bacteria - R & D for Food, Health and Livestock Purposes*. Vol i. InTech, 2013, 467–86.

Alves de Oliveira R, Komesu A, Vaz Rossell CE *et al.* Challenges and opportunities in lactic acid bioprocess design—From economic to production aspects. *Biochem Eng J* 2018;**133**:219–39.

Arif S, Batool A, Nazir W *et al.* Physiochemical Characteristics Nutritional Properties and Health Benefits of Sugarcane Juice. *Non-Alcoholic Beverages*. Elsevier, 2019, 227–57.

Basílio ACM, de Araújo PRL, de Morais JOF *et al.* Detection and Identification of Wild Yeast Contaminants of the Industrial Fuel Ethanol Fermentation Process. *Curr Microbiol* 2008;**56**:322–6.

Basso LC, Basso TO, Rocha SN. Ethanol Production in Brazil: The Industrial Process and Its Impact on Yeast Fermentation. In: Bernandes MA dos S (ed.). *Biofuel Production-Recent Developments and Prospects*. Vol 1530. IntechOpen, 2011, 85–100.

Basso TO, Gomes FS, Lopes ML *et al.* Homo- and heterofermentative lactobacilli differently affect sugarcane-based fuel ethanol fermentation. *Antonie Van Leeuwenhoek* 2014;**105**:169–77.

Comasio A, Harth H, Weckx S *et al.* The addition of citrate stimulates the production of acetoin and diacetyl by a citrate-positive *Lactobacillus crustorum* strain during wheat sourdough fermentation. *Int J Food Microbiol* 2019;**289**:88–105.

Duar RM, Lin XB, Zheng J *et al.* Lifestyles in transition: evolution and natural history of the genus *Lactobacillus*. *FEMS Microbiol Rev* 2017;**41**:S27–48.

Garofalo C, Osimani A, Milanović V *et al.* The occurrence of beer spoilage lactic acid bacteria in craft beer production. *J Food Sci* 2015;**80**:M2845–52.

Hickey M, Hillier A, Jago G. Metabolism of Pyruvate and Citrate in Lactobacilli. *Aust J Biol Sci* 1983;**36**:487.

Hugenholtz J. Citrate metabolism in lactic acid bacteria. *FEMS Microbiol Rev* 1993;**12**:165–78.

Kandler O. Carbohydrate metabolism in lactic acid bacteria. *Antonie Van Leeuwenhoek* 1983;**49**:209–24.

Kowalczyk M, Mayo B, Fernández M *et al.* Updates on metabolism in lactic acid bacteria in light of “Omic” technologies. In: Mozzi F, Raya RR, Vignolo GM (eds.). *Biotechnology of Lactic Acid Bacteria*. Chichester, UK: John Wiley & Sons, Ltd, 2015, 1–24.

Laëtitia G, Pascal D, Yann D. The citrate metabolism in homo- and heterofermentative LAB: A selective means of becoming dominant over other microorganisms in complex ecosystems. *Food Nutr Sci* 2014;**05**:953–69.

Lonvaud-Funel A. Lactic Acid Bacteria and Malolactic Fermentation in Wine. *Biotechnology of Lactic Acid Bacteria*. Chichester, UK: John Wiley & Sons, Ltd, 2015, 231–47.

Lucena BT, Dos Santos BM, Moreira JLS *et al.* Diversity of lactic acid bacteria of the bioethanol process. *BMC Microbiol* 2010;**10**:298.

Man JC de, Rogosa M, Sharpe ME. A medium for the cultivation of Lactobacilli. *J Appl Bacteriol* 1960;**23**:130–5.

Mendonça AA, da Silva PKN, Calazans TLS *et al. Lactobacillus vini*: Mechanistic response to stress by medium acidification. *Microbiol (United Kingdom)* 2019;**165**:26–36.

Passoth V, Blomqvist J, Schnürer J. *Dekkera bruxellensis* and *Lactobacillus vini* form a stable ethanol-producing consortium in a commercial alcohol production process. *Appl Environ Microbiol* 2007;**73**:4354–6.

Quintans NG, Blancato V, Repizo G *et al.* Citrate metabolism and aroma compound production in lactic acid bacteria. In: Mayo B, López P, Pérez-Martínez G (eds.). *Molecular Aspects of Lactic Acid Bacteria for Traditional and New Applications*. Vol 661. Kerala, 2008, 65–88.

Rodas AM, Chenoll E, Macián MC *et al. Lactobacillus vini* sp. nov., a wine lactic acid bacterium homofermentative for pentoses. *Int J Syst Evol Microbiol* 2006;**56**:513–7.

Rodas AM, Ferrer S, Pardo I. Polyphasic study of wine *Lactobacillus* strains: taxonomic implications. *Int J Syst Evol Microbiol* 2005;**55**:197–207.

Schell DJ, Dowe N, Ibsen KN *et al.* Contaminant occurrence, identification and control in a pilot-scale corn fiber to ethanol conversion process. *Bioresour Technol* 2007;**98**:2942–8.

Skinner K a, Leathers TD. Bacterial contaminants of fuel ethanol production. *J Ind Microbiol Biotechnol* 2004;**31**:401–8.

de Souza RB, de Menezes JAS, de Souza RFR *et al.* Mineral Composition of the Sugarcane Juice and Its Influence on the Ethanol Fermentation. *Appl Biochem Biotechnol* 2015;**175**: 209–222

de Souza RB, Santos BM dos, de Fátima Rodrigues de Souza R *et al.* The consequences of *Lactobacillus vini* and *Dekkera bruxellensis* as contaminants of the sugarcane-based ethanol fermentation. *J Ind Microbiol Biotechnol* 2012;**39**:1645–50.

Sun Z, Yu J, Dan T *et al.* Phylogenesis and Evolution of Lactic Acid Bacteria. In: Zhang H, Cai Y (eds.). *Lactic Acid Bacteria:* f*undamental and practice*. Dordrecht: Springer Science, 2014, 1–101.

Thanh VN, Mai LT, Tuan DA. Microbial diversity of traditional Vietnamese alcohol fermentation starters (banh men) as determined by PCR-mediated DGGE. *Int J Food Microbiol* 2008;**128**:268–73.

Tiukova I, Eberhard T, Passoth V. Interaction of *Lactobacillus vini* with the ethanol-producing yeasts *Dekkera bruxellensis* and *Saccharomyces cerevisiae*. *Biotechnol Appl Biochem* 2014;**61**:40–4.

Zhang W, Sun Z, Wu R *et al.* Comparative genome analysis of a new probiotic strain Lactobacillus casei Zhang. *Genomics II-Bacteria, Viruses and Metabolic Pathways*. 2013, 276–296.

Zhao XQ, Bai FW. Mechanisms of yeast stress tolerance and its manipulation for efficient fuel ethanol production. *J Biotechnol* 2009;**144**:23–30.

4.2 ARTIGO 2 Metabolismo de carboidratos de interesse industrial em *Lactobacillus vini*

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Resumo

A espécie *Lactobacillus vini* tem sido alvo de diversos estudos que compreendem: avaliação da sua influência na capacidade fermentativa e interação com leveduras do processo industrial; caracterização genética da linhagem *L. vini* JP7.8.9 através do sequenciamento do genoma, bem como através da identificação de mecanismos moleculares de resistência a antibióticos e a tolerância a acidificação do meio; análise fisiológica em resposta a composição nutricional dos substratos industriais. Entretanto, ainda não é possível compreender completamente como *L. vini* consegue competir com outros microrganismos na utilização das fontes de carbono disponíveis no meio. Portanto, o objetivo desse trabalho é analisar o perfil metabólico de *L. vini* em resposta as principais fontes de carbono presente no meio industrial. Parâmetros como cinética de consumo dos monossacarídeos (glicose e frutose) e dissacarídeos (sacarose, celobiose e lactose), e cinética de produção e rendimento de ácido lático foram analisados. Como resultado, a metabolização do carbono dos açúcares *Lactobacillus vini* tem como objetivo fundamental a produção de energia e a consequência imediata é o alto rendimento na produção de lactato. Mais da metade do carbono assimilados dos açúcares deve ser convertido a lactato por esta bactéria. Em substratos mistos, a glicose exerce forte efeito de repressão catabólica que uma vez cessada permite a pronta assimilação de dissacarídeos. Neste sentido, culturas estacionárias parecem funcionar como biotransformadoras de açúcar em lactato e a eficiência disto parece depender do fluxo de assimilação dos açúcares. Baixas velocidades de assimilação produzem altos índices de conversão. Esse fluxo parece ser negativamente regulados pela proteína RelA, semelhante ao que acontece em outros BALs e novos estudos devem ser realizados para se compreender como conciliar a atividade de RelA e a quantidade inicial de açúcar para se alcançar rendimentos mais altos possíveis.

Palavras-chave: fermentação lática, lactobacilos, metabolismo de carbono, rendimento.

Introdução

As bactérias ácido láticas (BAL) são os contaminantes bacterianos comumente encontrados na produção de etanol (BECKNER et al*.,* 2011; BASSO et al., 2014). Dentre estas, o gênero *Lactobacillus* sp. é o predominante (LUCENA et al., 2010; BECKNER et al*.,* 2011; MENDONÇA et al., 2019). O sucesso adaptativo deste gênero se deve a sua tolerância as condições do ambiente industrial de fermentação alcóolica, tais como a alta concentração de etanol, ao baixo pH e a condição anaeróbica, por exemplo (LUCENA et al., 2010; BECKNER et al*.,* 2011; MENDONÇA et al., 2019). Nesse contexto, a espécie *Lactobacillus vini* tem sido alvo de diversos estudos, principalmente por ter sido identificada como umas das principais BALs encontradas em destilarias da Suécia (PASSOTH et al., 2007) e do nordeste do Brasil (LUCENA et al., 2010). Esses estudos vão desde: avaliação da sua influência na capacidade fermentativa e interação com leveduras do processo industrial (DE SOUZA et al., 2012; TIUKOVA et al., 2014); sequenciamento do genoma (LUCENA et al., 2012); identificação de mecanismos moleculares de resistência a antibióticos e de tolerância a acidificação do meio (MENDONÇA et al., 2016; 2019) e análise fisiológica em resposta a composição nutricional do meio (DA SILVA et al., 2019). Estes estudos têm contribuído para a compreensão de como este lactobacilo consegue se manter no ambiente de fermentação alcóolica quando outras espécies são eliminadas do processo. Entretanto, ainda não é possível compreender completamente como *L. vini* consegue competir com outros microrganismos na utilização das fontes de carbono disponíveis no meio.

De forma geral, as BALs requerem meios ricos e complexos, para um crescimento significativo, tendo os açúcares como a principal fonte de carbono e energia (KOWALCZYK et al., 2016; PESSIONE et al., 2010). Durante a metabolização destes açúcares, bem como de outros compostos, são gerados intermediários que podem ser utilizados pela célula bacteriana como precursores em vias anabólicas e sustentam a produção de energia celular (KIM; GADD, 2008; PESSIONE et al., 2010). Tradicionalmente, a glicose e a lactose são os açúcares preferenciais e amplamente utilizados por bactérias láticas (WU; LU, 2014). Entretanto, outras hexoses também podem ser metabolizadas, bem como pentoses, e o perfil fisiológico de assimilação desses carboidratos pode variar entre as espécies desse gênero, o que pode ser justificado pela grande variação nos números de genes que codificam possíveis transportadores de açúcar.

A principal rota de importação dos açúcares do meio nessas bactérias depende de um sistema que acopla o transporte a fosforilação denominados de Sistema fosfotransferase (PTS

– phosphotransferase system), que usam o de fosfoenolpiruvato como doador de grupo fosfato (POSTMA et al., 1993; (KIM; GADD, 2008). Os sistemas PTS, em geral, são específicos para cada carboidrato e sua diversidade pode variar tanto entre as espécies quanto entre diferentes linhagens da mesma espécie (ZHANG et al., 2013). Por exemplo, as bactérias associadas à produção de laticínios apresentam uma maior dependência do PTS da lactose e uma menor diversidade de sistemas, enquanto as espécies de vida livre associadas a plantas podem apresentar uma diversidade maior de PTS, incluindo os β-glicosideos (ALEKSANDRZAK-PIEKARCZYK, 2013). O perfil de assimilação de cada açúcar e sua eficiência pode variar entre as espécies, correlacionado a abundância desses açúcares em seus ambientes naturais, indicando a sua adaptação a diferentes nichos ecológicos.

Na natureza além da capacidade de utilizar diversas fontes de carbono os organismos precisam ajustar seu metabolismo à disponibilidade nutricional (BOUTTE; CROSSON, 2013), onde a carência nutricional provoca alteração no metabolismo celular.. Em diversos organismo esse controle tem sido depende da produção de uma molécula conhecida com alarmônio produzida a partir de GTP (GEIGER; WOLZ, 2014). O acúmulo do alarmônio no citoplasma celular tem um efeito pleiotrópico nos organismos estudados. Em organismos modelo *Escherichia coli* e *Bacillus subtilis* os principais efeitos da resposta estringente envolve a desrrepressão dos genes de fontes secundárias de carbono e da resposta geral a estresse (BATTESTI et al*.,* 2011; GEIGER E WOLZ, 2014).

A produção do alarmônio é dependente da proteína codificada pelo gene *relA* ou *spoT*, ambos desempenhando a mesma atividade enzimática (TRAXLER et al*.,* 2008). Embora esses genes não sejam essênciais para sobrevivência da célula, são indispensáveis para sobrevida da população durante condições adversas de crescimento (KASAI et al., 2006; TRAXLER et al., 2008). A resposta estringente está intimamente relacionada ao regulon do gene *codY* (GEIGER; WOLZ, 2014). Em condições de abundância nutricional a resposta estringente é desativada, a produção do alarmônio diminui e o pool de GTP volta a aumentar (SONENSHEIN, 2005). O aumento da produção de energia também contribui com o aumento da produção do GTP que passa a refletir a abundância energética da célula. O GTP juntamente com os aminoácidos leucina, isoleucina e valina, são moduladores positivos do regulador *codY* que leva a repressão dos genes de metabolismo secundário de carbono, de resposta geral a estresse e de outras características celulares de fase estacionária (SONENSHEIN, 2005). Deste modo, a resposta estringente e a ativação do regulon de *codY* estão diretamente implicados na regulação do metabolismo energético da célula. *Lactobacillus vini* não apresenta em seu genoma o gene *codY* (LUCENA et al*.,* 2012). No entanto, dados de ontologia genética sugerem que o gene *pcrR* deve desempenhar sua função em *L. vini* (dados não publicados). Estes podem ser importantes componentes na sobrevivência de *L. vini* no ambiente industrial.

Portanto, o objetivo desse trabalho foi analisar o perfil metabólico de *L. vini* em resposta as principais fontes de carbono presentes no meio industrial. Parâmetros como cinética de consumo dos mono e dissacarídeos, cinética de produção e rendimento de ácido lático foram analisados. Compreender a fisiologia, pouco conhecida de *L. vini* JP7.8.9, permitirá a elucidação das características bioquímicas deste microrganismo. Esse estudo abre a perspectiva do emprego de *L. vini* na produção de metabólitos de alto valor agregado, como o ácido láctico, investigando quais os nutrientes são preferivelmente assimilados.

Material e Métodos

Microrganismos e condições de crescimento

A linhagem industrial *Lactobacillus vini* JP7.8.9, isolada da destilaria Japungú, Santa Rita, PB (LUCENA et al., 2010), e as linhagens mutantes *relA*$^-$ e *prcR*$^-$, foram cultivadas a 37 °C, em condições estáticas, em meio MRS Merck caldo (10 gL^{-1}peptona, 8 gL^{-1} extrato de carne, 4 gL^{-1} extrato de levedura, 20 gL^{-1} glicose, 5 gL^{-1} acetato de sódio, 1 gL^{-1} Tween 80, 2 gL^{-1} K_2HPO_4, 2 gL^{-1} Citrato de amônio, 0.2 gL^{-1} $MgSO_4$, 0.04 gL^{-1} $MnSO_4$) (DE SOUZA et al., 2012), ou meio sólido (contendo 2% de ágar) em jarra de anaerobiose com gerador de anaerobiose Anaerocult A® (Mikrobiologie). Para os cultivos das linhagens mutantes, o antibiótico eritromicina (Sigma Aldrich ®) foi adicionado ao meio, na concentração final de 5 µg/mL (MENDONÇA, 2018). Todas linhagens foram mantidas em estoque congelado a 15% de glicerol.

Análise de assimilação dos açúcares e crescimento celular em diferentes fontes de carbono

Para os ensaios de variação de fonte de carbono, o meio MRS foi reproduzido em laboratório a partir de seus componentes, segundo o fabricante (Merck), alterando-se os tipos de açúcar a serem testados (glicose, frutose, sacarose, celobiose e lactose) na concentração de 20 gL^{-1}. As células foram pré-cultivadas em meio MRS Merck e utilizadas para inocular os meios MRS-específicos para concentração inicial de 0,1 DO_{600nm}. Os cultivos foram conduzidos em sistema de batelada simples sem agitação durante 48 horas à 37ºC. As amostras coletadas foram utilizadas para leitura da absorbância em espectrofotômetro a 600nm. Em seguida foram centrifugadas e o sobrenadante reservado para análise de consumo dos açúcares e produção de metabólitos conforme protocolo descrito abaixo. Todas os cultivos foram realizados em duplicatas biológicas e triplicatas técnicas, e os resultados obtidos foram representados como médias simples (± desvio padrão).

Crescimento celular em duas fontes de carbono

As células de bactérias pré-cultivadas em meio MRS foram coletadas e re-suspensas para concentração celular inicial de 0,1 DO_{600nm} nos diferentes meios MRS reconstituídos, contendo agora, como fontes de carbono: (A) glicose 10 gL^{-1} + sacarose 10 gL^{-1} e (B) glicose 10 gL^{-1} + celobiose 10 gL^{-1}, totalizando 20 gL^{-1} de açúcares totais em cada meio (A e B). Os cultivos foram conduzidos em sistema de batelada simples sem agitação durante 24 horas à 37ºC. As amostras foram coletadas e analisadas conforme descrito acima.

Indução do crescimento em meio contendo lactose

O meio MRS foi reproduzido conforme fabricante, e a lactose foi utilizada como fonte de carbono, em substituição à glicose. As células bacterianas foram cultivadas em meio MRS comercial, coletadas e re-suspensas para concentração celular inicial de 0,1 DO_{600nm} em meio MRS reconstituído, contendo 20 gL^{-1} de lactose, ou 20 gL^{-1} de lactose com adição de 0,2 gL^{-1} de glicose, ou 20 gL^{-1} de lactose 0,2 gL^{-1} de celobiose. Os cultivos foram realizados sem agitação durante 48 horas à 37ºC. As amostras coletadas e analisadas conforme descrito acima.

Análise metabólica

As amostras coletadas ao longo das incubações foram centrifugadas para coleta do sobrenadante, que foi filtrado em membrana 0,22mm, para determinação da concentração do açúcar residual e dos metabólitos produzidos, por cromatografia líquida de alta pressão (HPLC) em equipamento Agilent Technologies 1200 Series com a coluna modelo HPX87H (BioRad) em fase móvel de ácido sulfúrico a 5 mM, a 45ºC. A identificação dos metabólitos foi feita por comparação dos tempos de retenção com soluções-padrão e a concentração de cada metabólito foi determinada por integração de picos do cromatograma e relação da área de cada pico com o fator de calibração de cada solução-padrão, através da metodologia de ZHAO et *al*., (2009) adaptada por DE SOUZA et al*.,* 2012.

Resultados e Discussão

Crescimento celular em diferentes fontes de carbono

Para a avaliação da cinética de crescimento, as células de *L. vini* JP7.8.9 foram inoculadas em meio MRS reconstituído variando a fonte de carbono. Esses experimentos foram realizados em condições estáticas, minimizando assim a oxigenação do meio, já que o crescimento desta bactéria em aerobiose é muito lento (dado não mostrado). Foram avaliados os açúcares presentes no mosto de fermentação de cana de açúcar (sacarose e seus produtos de hidrólise glicose e frutose) e dissacarídeos de importância industrial (celobiose da hidrólise da celulose e lactose dos produtos lácteos). O meio com glicose foi utilizado como referência.

Os açúcares são a principal fonte de carbono e energia para as BALs que são cultivadas para a produção de alimentos e rações fermentados, bem como em meios laboratoriais (KOWALCZYK et al., 2016). No intervalo de 48 horas foi observado um baixo crescimento da bactéria no meio contendo lactose como fonte de carbono (Figura 1). A lactose é um dissacarídeo formado por uma glicose e uma galactose. Sua metabolização é iniciada com a quebra da ligação β-1,4 pela enzima β-galactosidase. Os monômeros resultantes são degradados, a glicose diretamente pela via glicolítica e a galactose sendo metabolizada pela via da tagatose 6-fosfato, ou alternativamente pela via de Leloir (MONEDERO et al., 2007). Trabalhos anteriores (dados não publicados) mostram que a linhagem JP7.8.9 não apresenta deficiência na assimilação de nenhum desses dois monômeros. Portanto, pode-se presumir que o acúmulo de glicose resultante da hidrólise da lactose deva exercer efeito de repressão catabólica sobre o operon *lac* produzido pela ligação da proteína repressora semelhante a CcpA/P-Ser-HPr como acontece em *L. casei* (MONEDERO et al., 2007). Já a sacarose e a celobiose permitiram crescimento celular com a mesma eficiência da glicose e da frutose (Figura 1; Tabela 2). Sacarose é um dissacarídeo formado por uma molécula de glicose e uma molécula de frutose com ligação α-1,2-frutofuranosídeo, enquanto que celobiose é um dissacarídeo formado por duas moléculas de glicose unidas por ligação β-1,4-glicosídica. Seguindo a lógica acima, é possível sugerir que não há em *L. vini* o mecanismo de repressão catabólica ou mesmo de inibição alostérica das enzimas que representam o primeiro passo na metabolização desses açúcares.

Até o momento, a caracterização do transporte de glicose em *L. vini* não foi reportada, mas o transporte de glicose em *Lactococcus lactis*, uma bactéria filogeneticamente relacionada com *L. vini*, foi elucidado. Em *L. lactis* existem dois sistemas de transporte do tipo manose-

PTS (PTSMan) e celobiose-PTS (PTSCel) e um sistema de transporte ativo secundário (GlcU) (CASTRO et al., 2009). A bactéria *L. vini* JP7.8.9 contém no seu genoma operons do metabolismo de celobiose e sacarose, e grande quantidade de genes que codificam para β-glicosidases, além de sistemas específicos celobiose-PTS e sacarose-PTS (LUCENA et al., 2012), o que deve propiciar o melhor crescimento de *L. vini* JP7.8.9 em meio contendo sacarose ou celobiose, quando comparado com a glicose. De fato, observa-se o crescimento mais pronunciado em sacarose e celobiose do que em glicose (Figura 1). O sistema de transporte PTS é tido como o principal translocador de glicose em muitas bactérias do filo *Firmicutes* ao qual pertence à família *Lactobacillaceae* (JAHREIS et al., 2008). Assim sendo, é válido assumir que em *L. vini* a sacarose e a celobiose são transportadas e concomitantemente fosforiladas pelos sistemas PTS.

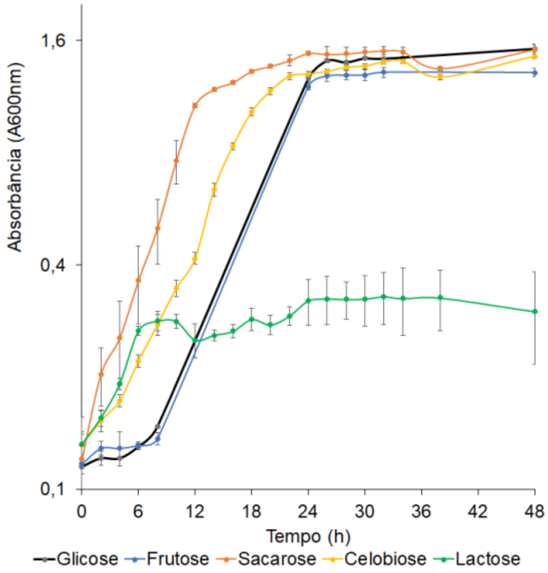


Figura 1. Perfil de crescimento de culturas de *Lactobacillusn vini* JP7.8.9 em meio MRS reconstituído contendo diferentes açúcares.

Em seguida foram avaliados os metabólitos das culturas bacterianas nos diferentes açúcares testados (Figura 2). Neste caso, não foram apresentados os dados da lactose porque não houve consumo do açúcar e, consequentemente, não houve produção de lactato. Glicose foi completamente consumida em 48 h de cultivo, o que não houve com os demais açúcares. No entanto, a produção final de lactato foi em torno de 10 g/L para todos os açúcares (Figura 2). O maior rendimento final de lactato foi atingido em sacarose (>0,8 g/g), enquanto que o menor rendimento final foi atingido em glicose (<0,6 g/g). Isto indicaria que a maior parte do carbono proveniente da glicose seria assimilada para a formação de biomassa. Contudo, as culturas atingem a mesma biomassa com os diferentes açúcares (Figura 1), o que indicaria que o carbono da glicose seria dissimilado como um subproduto do crescimento. No entanto, nenhum outro metabólito foi detectado nas culturas de *L. vini* em concentrações que explicariam essa diferença. Portanto, sugerimos que o metabolismo celular em glicose deva induzir vias anabólicas que promovem produção e consequente dissimilação de carbono na forma de CO_2, tais como a via da tagaturose 6-fosfato. O maior rendimento alcançado foi de 1 g/g no tempo de 12 h de cultivo em sacarose (Figura 2), quando a cultura já está no final da fase exponencial de crescimento e entrando na fase estacionária (Figura 1). Isto significa que todo carbono da sacarose é convertido a lactato na estequiometria de 1 mol de sacarose para 4 moles de lactato, produzindo 4 moles de ATP pela fosforilação a nível de substrato pela via glicolítica. A partir desse ponto verifica-se a desconexão entre o consumo de açúcar que se mantém ativo, produzindo lactato e ATP, e a produção de biomassa que é paralisada. Sugere-se que no caso dos lactobacilos homofermentadores, como é o caso de *L. vini*, a entrada na fase estacionária é devida fundamentalmente à produção de lactato e não à escassez de nutrientes. De fato, isto foi comprovado neste trabalho, já que ainda há açúcar residual em todas as condições testadas (Figura 2). No ponto de 24 h que representa a parada de crescimento, a menor concentração de lactato detectada foi de 5 g/L (55 mmol/L) no meio com frutose. Embora esta concentração esteja acima do valor de concentração inibitória mínima (CIM) para este composto, ainda assim tem efeito desacelerador no crescimento de *L. vini* (MENDONÇA et al., 2019). Portanto, é possível se supor que o metabolismo celular se mantém em pleno funcionamento pela manutenção do consumo de açúcares sem que haja crescimento celular justamente pela produção de lactato. O ATP produzido pela via glicolítica seria, portanto, completamente utilizado pelos mecanismos de bombas de extrusão para a detoxificação celular do lactato. Esta característica pode ser futuramente explorada pela utilização de *L. vini* como uma usina de conversão simples de açúcares a lactato em substratos ricos em sacarose, como caldo de cana, sem perda de massa na produção de biomassa ou de carbono pela dissimilação em CO_2 ou outros produtos de fermentação. Lactato é um importante metabólito de interesse industrial para síntese de polímeros biodegradáveis (ALVES DE OLIVEIRA et al., 2018).

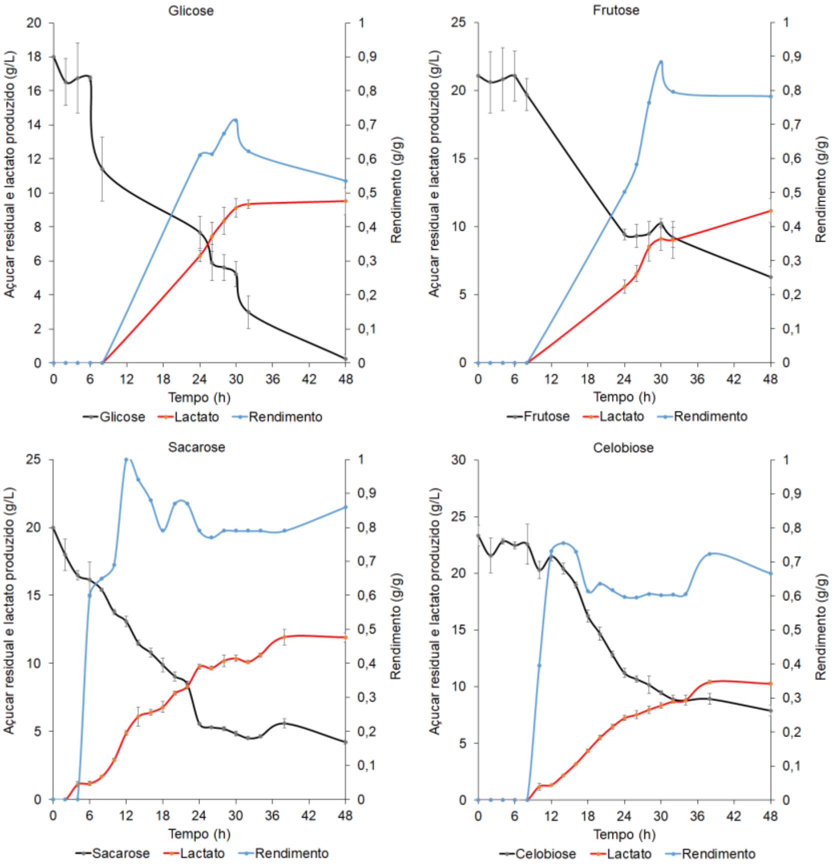


Figura 2. Perfil do consumo de açúcares e da produção de lactato e seu rendimento em culturas de *Lactobacillus vini* JP7.8.9 em meio MRS reconstituído contendo diferentes açúcares.

Um fato bastante relevante apresentado no presente trabalho foi a eficiência de *L. vini* na captação e conversão de celobiose. Este açúcar é derivado da hidrólise enzimática parcial de biomassas celulósicas gerada para produção etanol de segunda geração (ZABED et al., 2017). O pico de rendimento de conversão de 0,75 g/g (Figura 2) foi observado em 12 h de crescimento, no meio da fase exponencial (Figura 1). No entanto, o consumo do açúcar foi muito baixo, com baixa produção de lactato. Nesse caso, a maior eficiência global foi alcançada no tempo de 38 h, com o maior ponto de produção de lactato e maior consumo de celobiose, mas com baixa produtividade volumétrica de 0,29 g de lactato/L.h para esse açúcar. Além disso, no perfil de metabólitos das culturas com celobiose foi observado um pico cromatográfico em tempo de retenção semelhante ao da glicose (dados não mostrados). Isto indica que parte da massa de celobiose estaria voltando para o meio durante o transporte pelo sistema PTSCel. Um fenômeno deste tipo foi observado em *L. sanfrancisco* em relação a captação de maltose, um dissacarídeo de glicose como a celobiose. Nesse caso, a capacidade de hidrolise do dissacarídeo excede a capacidade de assimilação da glicose pela via glicolítica e a glicose excedente é liberada de volta ao meio como forma de se evitar a presença de glicose livre no interior celular que poderia disparar uma sinalização de repressão catabólica generalizada (NEUBAUER et al., 1994). Isto pode explicar o período de queda do rendimento em lactato no inicial da fase estacionária.

Análise de fluxo metabólico e papel da proteína RelA

Para compreensão dos possíveis efeitos do mecanismo de repressão catabólica, uma linhagem derivada da JP7.8.9 com deleção de genes homólogos a *prcR* e *relA* foram testadas em cultivos com glicose (Figura 3). Os resultados mostraram que a linhagem *relA*$^-$ não é capaz de esgotar o açúcar do meio, com menor produção global de lactato e menor rendimento final deste metabólito (Figura 3). Adicionalmente, as células foram cultivadas em meio contendo a mistura de glicose e sacarose e de glicose e celobiose para se analisar o possível efeito da glicose sobre a assimilação dos dissacarídeos (Figura 4). Em todas as condições testadas houve o total consumo da glicose no período de 12 h de cultivo, com baixo consumo dos dissacarídeos tanto pela linhagem parental JP7.8.9 como pelos seus derivados mutantes (Figura 4). Depois desse ponto ocorre o consumo dos dissacarídeos residuais, que correspondem a quase totalidade dos dissacarídeos adicionados no meio. A sacarose residual foi praticamente a mesma para as três linhagens, enquanto que a parental apresentou consumo um pouco maior da celobiose (Figura 4).

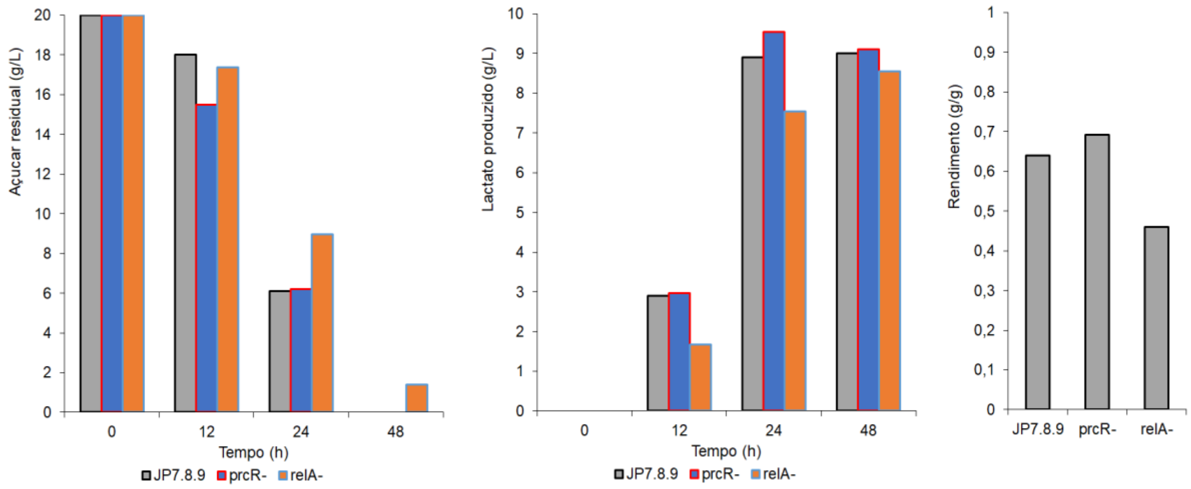


Figura 3. Perfil de consumo de açúcar, de produção de lactato e do rendimento final da linhagem parental *Lactobacillus vini* JP7.8.9 e de seus derivados mutantes para os genes *relA* e *prcR* cultivadas em meio MRS contendo glicose.

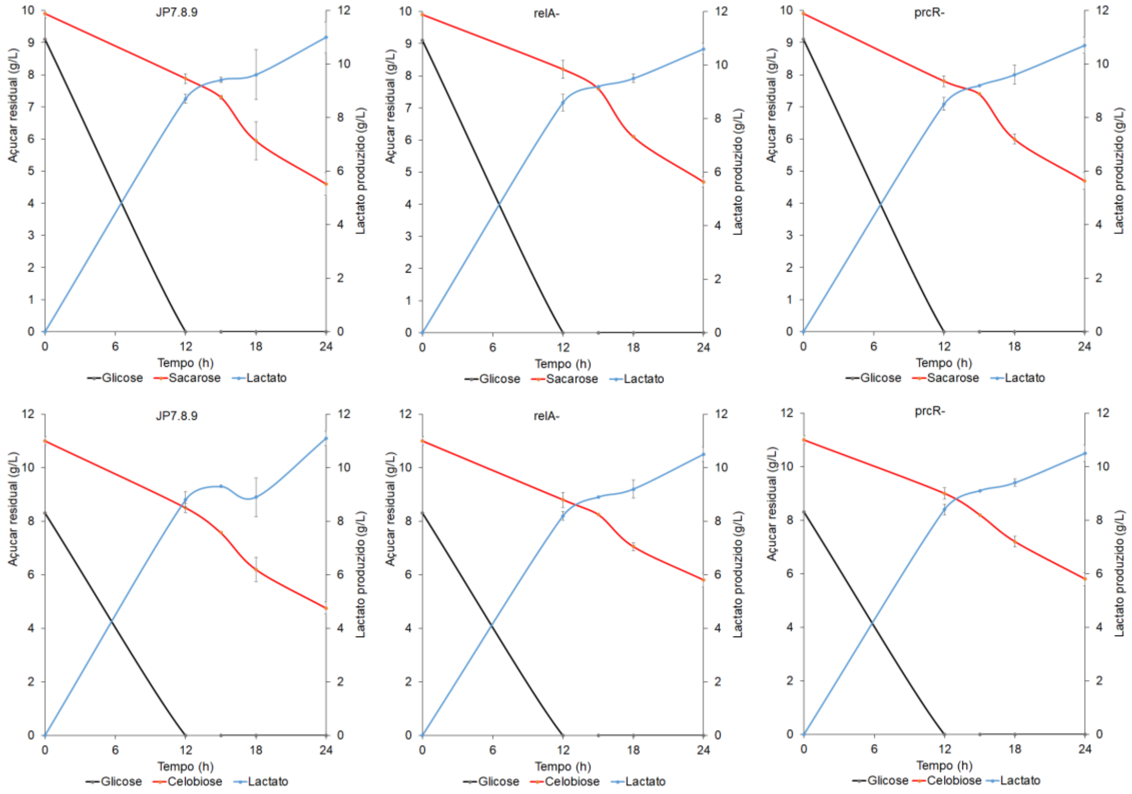


Figura 4. Perfil de consumo de açúcar e de produção de lactato da linhagem parental *Lactobacillus vini* JP7.8.9 e de seus derivados mutantes para os genes *relA* e *prcR* cultivadas em meio MRS contendo mistura de glicose+sacarose e glicose+celobiose.

Considerando-se apenas a segunda fase dos cultivos, foi possível a realização de cálculos de velocidade de consumo e de quantidade de carbono do açúcar consumido e do carbono convertido a lactato (Tabela 1). Os dados calculados para o mutante *prcR* foram praticamente os mesmos da linhagem parental. O primeiro fato a se considerar é que a velocidade de produção de lactato de 192 mmol/L.h foi constante, independente da linhagem e do dissacarídeo, indicando que as diferenças no rendimento de lactato se devem as etapas metabólicas anteriores. De fato, verificou-se que a velocidade de consumo dos açucares foi diferente, resultando em diferenças na quantidade de carbono assimilado e na quantidade de carbono do açúcar que foi destinado para a produção de lactato (Tabela 1). Na linhagem parental, a conversão do carbono assimilado a lactato foi semelhante para os dois dissacarídeos. Isso indica que o fluxo da via glicolítica deve ser constante. No entanto, tanto a velocidade de consumo da celobiose como a quantidade de carbono assimilado da celobiose foram cerca de 15% maior do que esses parâmetros em sacarose, sem que isso refletisse na produção de lactato. Consequentemente, o índice de conversão de carbono da sacarose em carbono de lactato foi de 0,67 e da celobiose a lactato foi de 0,58. Portanto, isto indica o desperdício de carbono da celobiose e a explicação para isso deve estar relacionada com a saída de uma de parte da glicose liberada pela hidrólise da celobiose, como apontado acima. Na linhagem *relA*$^-$ esse índice de conversão a partir da sacarose foi de 0,55 e foi muito maior como 0,95 no caso da celobiose.

Tabela 1. Valores de velocidade de consumo (*r*S) e de produção de lactato (*r*P) e valores correspondentes de mmol de carbono consumidos e convertidos a lactato nas linhagens de *Lactobacillus vini* JP7.8.9 e seu mutante *relA*$^-$ na segunda fase dos cultivos em meios com glicose+sacarose e glicose+celobiose.

		Consumo açúcar		Produção lactato		
Açúcar	Linhagem	*r*S (mmol/L.h)	mmolC	*r*P (mmol/L.h)	mmolC	ICC*
Sacarose	JP7.8.9	273	115	192	77	0,67
	relA$^-$	291	122	192	67	0,55
Celobiose	JP7.8.9	312	132	192	77	0,58
	relA$^-$	250	116	192	110	0,95

*ICC= índice de conversão de carbono (mmolC lactato/mmolC açúcar consumido)

A assimilação desses dois dissacarídeos se faz a partir de sistemas PTS de fosforilação, gerando uma molécula de glicose fosforilada e uma hexose não fosforilada, frutose no caso da sacarose e glicose no caso da celobiose (SIRAGUSA et al., 2014). Nesse caso, os dados sugerem que a ausência da proteína RelA na linhagem mutante estaria diminuindo um pouco utilização da frutose para lactato em relação ao parental. Por outro lado, a ausência de RelA promoveria maior aproveitamento da glicose não fosforilada.

A eficiência de conversão de carbono do açúcar foi relacionada com a velocidade de consumo do açúcar a partir de uma simulação matemática usando os dados da Tabela 1. Foi observado uma relação inversa entre esses dois parâmetros indicando que quanto mais rápido a célula consome açúcar menos carbono é desviado para a produção de lactato (Figura 5). Desta maneira, é possível sugerir que a proteína RelA atuaria como um sensor no transporte de celobiose, evitando que a glicose excedente gere quantidades letais de lactato se acumulando no meio de cultura. Isso explicaria a queda no rendimento de lactato no decorrer dos cultivos (Figura 2). Esta simulação também indicou os limites metabólicos nesses parâmetros (Figura 5). Abaixo do valor de 448 mmol de açúcar/L.h a totalidade dos carbonos dos açúcares seria convertida a lactato; já a partir de 480 mmol de açúcar/L.h o metabolismo atingiria o menor índice de conversão de 0,56 e as células tenderiam a manter esse índice mesmo que houvesse excedente de açúcar no meio.

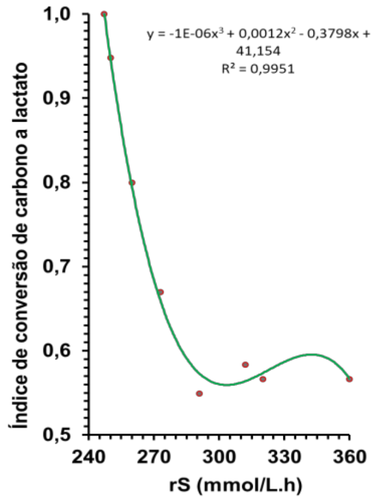


Figura 5. Relação entre o índice de conversão de carbono e a velocidade de assimilação de açúcar em *Lactobacillus vini* JP7.8.9

Consequentemente, possíveis avanços tecnológicos no sentido de se utilizar esta bactéria como produtora de lactato teriam que levar em consideração as concentrações adequadas de açúcares nos substratos de fermentação de maneira a se assegurar da máxima conversão com o mínimo de desperdícios na forma de açúcar residual.

Ativação do sistema de transporte de lactose em *L. vini*

Como mostrado na Figura 1, a lactose é pouco consumida por esta bactéria. O crescimento inicial parece ser devido ao efeito do pré-inóculo das células em meio MRS contendo glicose. Isto indica que o sistema de transporte desse dissacarídeo necessita ser ativado por outro açúcar que não a própria lactose. Este fenômeno foi relatado para a bactéria *Lactococcus lactis* na qual o transporte de lactose foi ativado pela presença de quantidades muito pequenas de celobiose no meio de cultura (ALEKSANDRCZYK-PIEKARCZYK et al., 2005). Diante dessas evidências, cultivos foram realizados com a linhagem parental e os dois mutantes em meio MRS reconstituído contendo 20 g/L de lactose e 0,2 g/L de glicose ou de celobiose como indutores (Figura 6).

Os resultados confirmaram o baixo crescimento da linhagem parental JP7.8.9 em meio contendo apenas lactose. Nessa condição não houve consumo da lactose, confirmando que o pequeno crescimento inicial deve ser devido aos efeitos do pré-inóculo. Quando um dos dois indutores foi adicionado ao meio houve indução do crescimento celular com consumo de lactose do meio. Os resultados foram semelhantes para os dois indutores e entre as duas linhagens mutante *relA*$^-$ e *prcR*$^-$ (Figura 6). O período de crescimento foi mais extenso em lactose (Figura 6) do que nas outras fontes de carbono testadas (Figura 1), o que indica que a velocidade de crescimento neste dissacarídeo é menor do que nos demais açúcares. Apesar dos perfis de crescimento serem semelhantes entre as linhagens, o consumo de lactose é maior na linhagem parental (Tabela 2). O consumo de lactose e a produção de lactato foram observados durante a fase exponencial de crescimento (Figura 6), e este estado dinâmico não permite cálculos de velocidades sem levar em consideração a taxa de crescimento específico das culturas. Isto difere do estado estacionário observado para os meios com sacarose e celobiose (Figura 4). O rendimento final em massa apresentou valores bastante altos (>0,8), a semelhança do que ocorreu para os cultivos em sacarose (Figura 2). Isso mostra que a conversão de lactose a lactato também acontece de maneira bastante eficiente (Tabela 2). No entanto, o gargalo para esse tipo de produção se refere ao fato que o metabolismo mais lento da lactose resulta em baixa velocidade volumétrica, ou seja, maior tempo de fermentação. Outro fator relevante é o valor do açúcar residual (Figura 6) que foi próximo aos observados para os outros dois dissacarídeos, sacarose e celobiose (Figura 2).

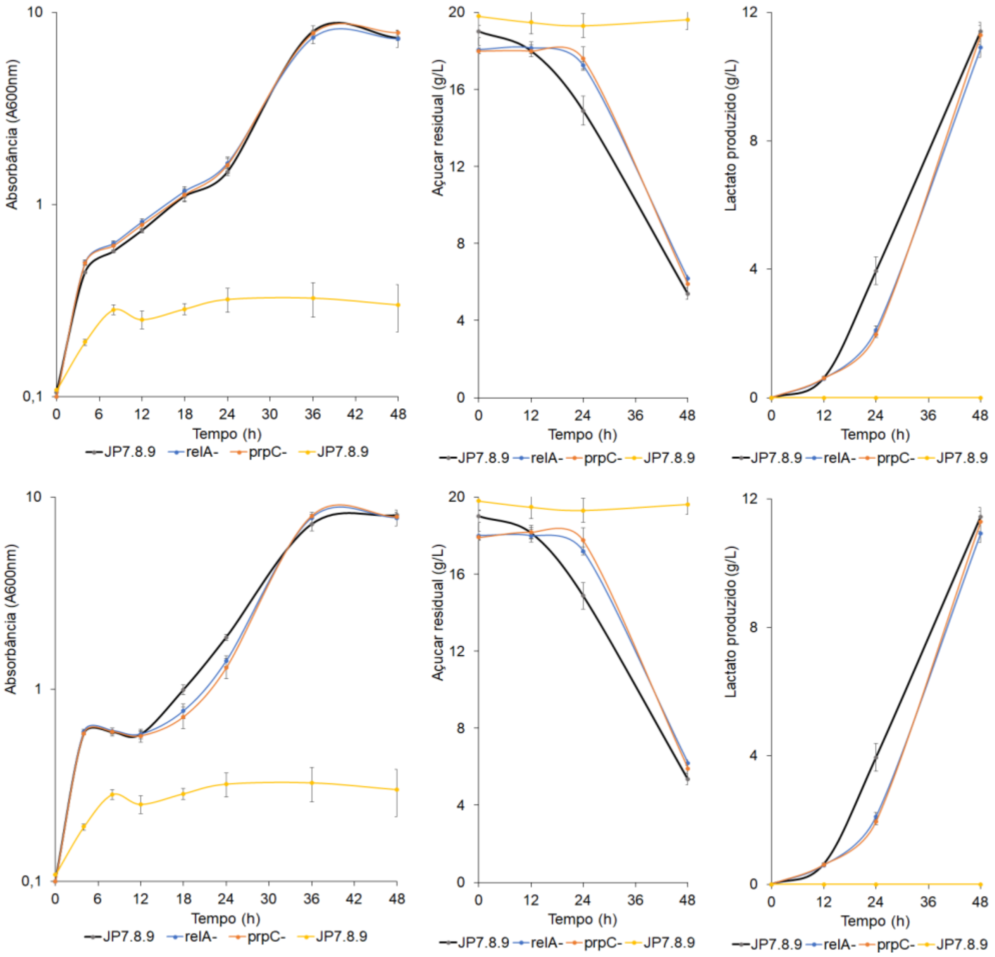


Figura 6. Perfil de crescimento, açúcar consumido e lactato produzido em cultivos da linhagem parental *Lactobacillus vini* JP7.8.9 e seus mutantes *relA*$^-$ e *prcR*$^-$ em meio MRS contendo lactose suplementado com glicose (painéis superiores) ou celobiose (painéis inferiores). O cultivo da linhagem parental em meio contendo apenas lactose (linhas em amarelo) estão mostrados.

O índice de conversão de carbono na faixa de 0,75 foi igual para as três linhagens (Tabela 2), e superior aos calculados para a sacarose e celobiose (Tabela 1). O sistema PTSLac também internaliza a lactose fosforilando a glicose, e liberando uma molécula de galactose não fosforilada que deve ser metabolizada pela via de Leloir. Os valores de conversão calculados para as células mutantes indicam, portanto, que as proteínas RelA e PrcR não estão relacionadas com o metabolismo da galactose não fosforilada que é liberada da hidrólise da lactose. Neste caso, o gargalo da assimilação da lactose deve estar relacionado a baixa velocidade de consumo do açúcar ou ao baixo fluxo de metabolização da galactose pela via de Leloir.

Tabela 2. Valores médios dos parâmetros fermentativos de culturas de *Lactobacillus vini* JP7.8.9 e seus mutantes *relA*$^-$ e *prcR*$^-$ em meio MRS contendo lactose suplementado com glicose ou celobiose.

Linhagem	Lactose consumida (g/L)	Rendimento (g/g)	ICC*
JP7.8.9	13,62 ± 0,02	0,84 ± 0,0003	0,74
relA$^-$	11,84 ± 0,04	0,92 ± 0,0019	0,76
prcR$^-$	12,05 ± 0,05	0,94 ± 0,0037	0,75

*ICC= índice de conversão de carbono (mmolC lactato/mmolC açúcar consumido)

Conclusão

Em *Lactobacillus vini* a metabolização do carbono dos açúcares tem como objetivo fundamental a produção de energia e a consequencia imediata é o alto rendimento na produção de lactato. Pelo menos metade do carbono assimilados dos açúcares deve ser convertido a lactato por esta bactéria. Em substratos mistos, a glicose exerce forte efeito de repressão catabólica que uma vez cessada permite a pronta assimilação de dissacarídeos. Neste sentido, culturas estacionárias parecem funcionar como biotransformadoras de açúcar em lactato e a eficiência disto parece depender do fluxo de assimilação dos açúcares. Baixas velocidades de assimilação produzem altos índices de conversão. Esse fluxo parece ser negativamente regulados pela proteína RelA, semelhante ao que acontece em outros BALs e novos estudos devem ser realizados para se compreender como conciliar a atividade de RelA e a quantidade inicial de açúcar para se alcançar rendimentos mais altos possíveis.

Referências

ALEKSANDRZAK-PIEKARCZYK, T.; et al. **Alternative Lactose Catabolic Pathway in *Lactococcus lactis* IL1403**. Applied and environmental microbiology. 71(10): 6060 – 6069. 2005.

ALEKSANDRZAK-PIEKARCZYK, T. **Lactose and β-glucosides metabolism and its regulation in *Lactococcus lactis***: a review. In: KONGO, M. (ed). Lactic Acid Bacteria - R& D for Food, Health and Livestock Purposes. InTechOpen. vol I. 467 – 486. 2013.

ALVES DE OLIVEIRA, R. et al. **Challenges and opportunities in lactic acid bioprocess design –** From economic to production aspects. Biochem Eng J. 133: 219 – 239. 2018.

BASSO, T. O. et al. **Homo- and heterofermentative lactobacilli differently affect sugarcane-based fuel ethanol fermentation**. Antonie van Leeuwenhoek.105:169–177. 2014.

BATTESTI, A.; MAJDALANI, N.; GOTTESMAN, S. **The RpoS-Mediated General Stress Response in *Escherichia coli***. Annu Rev Microbiol. 65: 189 – 213. 2011.

BECKNER, M.; IVEY, M. L.; PHISTER, T. G. **Microbial contamination of fuel ethanol fermentations**. Lett Appl Microbiol. 53: 387 – 394. 2011.

BOUTTE, C. C.; CROSSON, S. **Bacterial lifestyle shapes stringent response activation**. Trends in Microbiol. 21: 174 – 80. 2013.

CASTRO, R. et al. **Characterization of the individual glucose uptake systems of *Lactococcus lactis*: mannose-PTS, cellobiose-PTS and the novel GlcU permease**. Molecular Microbiology. 71: 795–806. 2009.

DA SILVA, P. K. N. et al. **Nutritional requirement for *Lactobacillus vini* growth in sugarcane derivative substrate of ethanol fermentation**. FEMS Microbiology Letters. 366(16): fnz202. 2019.

DE SOUZA, R. B. et al. **The consequences of *Lactobacillus vini* and *Dekkera bruxellensis* as contaminants of the sugarcane-based ethanol fermentation**. J Ind Microbiol Biotechnol. 39(11): 1645 – 1650. 2012.

GEIGER, T.; WOLZ, C. **Intersection of the stringent response and the CodY regulon in low GC Gram-positive bacteria**. International Journal of Medical Microbiology. 304(2): 150 – 5. 2014.

JAHREIS, K. et al. **Ins and outs of glucose transport systems in eubacteria**. FEMS Microbiological Review. 32: 891-907. 2008.

KASAI, K. et al. **Physiological analysis of the stringent response elicited in an extreme thermophilic bacterium, *Thermus thermophilus***. J Bacteriol. 188(20): 7111 – 7122. 2006.

KIM, B. H.; GADD, G. M. **Bacterial physiology and metabolism**. Cambridge: Cambridge University Press. 2008.

KOWALCZYK, M. et al. **Updates on Metabolism in Lactic Acid Bacteria in Light of "Omic" Technologies**. In: MOZZI, F.; RAYA, R.R; VIGNOLO. G.M. (eds) Biotechnology of Lactic Acid Bacteria: Novel Applications, 2nd Ed. West Sussex: Wiley-Blackwell. p 1 – 24. 2016.

LUCENA, B. T. L. et al. **Diversity of Lactic Acid Bacteria of the bioethanol process**. BMC Microbiol. p 10: 298. 2010.

LUCENA, B. T. L. et al. **Genome Sequences of the Ethanol-Tolerant *Lactobacillus vini* Strains LMG 23202T and JP7.8.9**. J Bacteriol. p 194(11): 3018. 2012

MENDONÇA, A. A. et al. **First identification of Tn916-like element in industrial strains of *Lactobacillus vini* that spread the tet-M resistance gene**. FEMS Microbiol Lett. 363(3): fnv240. 2016.

MENDONÇA, A.A. **Aspectos fisiológicos e genéticos da bactéria *Lactobacillus vini* em condições de estresse.** 2018. 185 f. Tese (Doutorado) – Curso de Pós-graduação em Genética, Departamento de Genética, Universidade Federal de Pernambuco, Recife, 2018.

MENDONÇA, A. A. et al. ***Lactobacillus vini:* mechanistic response to stress by medium acidification**. Microbiology. 165(1): 26 – 36. 2019.

MONEDERO, V. et al. **The Phosphotransferase System of *Lactobacillus casei*:** Regulation of Carbon Metabolism and Connection to Cold Shock Response. Journal of Molecular Microbiology and Biotechnology. 12: 20-32. 2007.

NEUBAUER, H. et al. **Mechanism of maltose uptake and glucose excretion in *Lactobacillus sanfrancisco***. Journal of Bacteriology. 176(10): 3007 – 3012. 1994.

PASSOTH, V.; BLOMQVIST, J.; SCHUNÜRER, J. ***Dekkera bruxellensis* and *Lactobacillus vini* Form a Stable Ethanol-Producing Consortium in a Commercial Alcohol Production Process**. Applied Environmental Microbiology. 73(13): 4354 – 4356. 2007.

PESSIONE, A.; LAMBERTI, C.; PESSIONE, E. **Proteomics as a tool for studying energy metabolism in lactic acid bacteria**. Mol Biosyst. 6(8): 1419 – 1430. 2010.

POSTMA, P. W.; LENGELER, J. W.; JACOBSON, G. R. **Phosphoenolpyruvate: carbohydrate phosphotransferase systems of bacteria**. Microbiol Rev. 57(3): 543 – 594. 1993.

SIRAGUSA, S. et al. **Fermentation and proteome profiles of *Lactobacillus plantarum* strains during growth under food-like conditions**. Journal of Proteomics. 366 – 380. 2014.

SONENSHEIN, A.L. ***CodY*, a global regulator of stationary phase and virulence in Gram-positive bacteria**. Current Opinion in Microbiology. 8: 203 – 207. 2005.

TIUKOVA, I.; EBERHARD, T.; PASSOTH, V. **Interaction of *Lactobacillus vini* with the ethanol-producing yeasts *Dekkera bruxellensis* and *Saccharomyces cerevisiae***. Biotechnol Appl Biochem. 61: 40 – 4. 2014.

TRAXLER, M.F. et al. **The global, ppGpp-mediated stringent response to amino acid starvation in *Escherichia coli***. Mol Microbioly. 68(5): 1128 – 48. 2008.

WU, R.; LU, J. **Proteomics of Lactic Acid Bacteria** In: Lactic Acid Bacteria: fundamental and practice. Zhang, H.; Cai, Y. (Eds) Dordrecht: Springer Science. p 249 – 301. 2014.

ZABED, H. et al. **Bioethanol production from renewable sources:** Current perspectives and technological progress. Renewable and Sustainable Energy Reviews. 71: 475 – 501. 2017.

ZHANG, W. et al. **Comparative genome analysis of probiotic *Lactobacillus casei Zhang***. In: Genomics II: bacteria, viruses and metabolic pathways. 1st ed. Hong Kong: iConcept Press Ltd. p. 276 – 296. 2013.

ZHAO, X. Q. et al. **Impact of zinc supplementation on the improvement of ethanol tolerance and yield of selfflocculating yeast in continuous ethanol fermentation. Journal of Biotechnology**. 139: 55–60. 2009.

5 CONCLUSÃO

Este estudo mostrou que o crescimento limitado de *Lactobacillus vini* em substratos industriais, derivados da cana-de-açúcar, é consequência da falta de oligopeptídeos / aminoácidos e citrato como requisitos nutricionais essenciais. Essa limitação pode ser superada pela lise de parte da população microbiana, (principalmente células de levedura), que liberam esses suplementos no substrato e permitem o crescimento de células bacterianas durante o processo de fermentação. Também consolida a metabolização do citrato através do piruvato, fornecendo acetato suficiente para a formação da biomassa. Também foi possível concluir que em *L. vini* a metabolização do carbono dos açúcares tem como objetivo fundamental a produção de energia e a consequencia imediata é o alto rendimento na produção de lactato. Pelo menos metade do carbono assimilados dos açúcares deve ser convertido a lactato por esta bactéria. Em substratos mistos, a glicose exerce forte efeito de repressão catabólica que uma vez cessada permite a pronta assimilação de dissacarídeos. Neste sentido, culturas estacionárias parecem funcionar como biotransformadoras de açúcar em lactato e a eficiência disto parece depender do fluxo de assimilação dos açúcares. Baixas velocidades de assimilação produzem altos índices de conversão. Esse fluxo parece ser negativamente regulados pela proteína RelA, semelhante ao que acontece em outros BALs e novos estudos devem ser realizados para se compreender como conciliar a atividade de RelA e a quantidade inicial de açúcar para se alcançar rendimentos mais altos possíveis. Essas informações sobre as características metabólicas de *L. vini* revelam aspectos significativos de seu desempenho em processos de fermentação industrial.

REFERÊNCIAS

ADAMS, M. R. **Safety of industrial lactic acid bacteria**. Journal of Biotechnology. 68: 171–178. 1999.

ALEKSANDRZAK-PIEKARCZYK, T.; et al. **Alternative Lactose Catabolic Pathway in *Lactococcus lactis* IL1403**. Applied and environmental microbiology. 71(10): 6060 – 6069. 2005.

ALEKSANDRZAK-PIEKARCZYK, T. **Lactose and β-glucosides metabolism and its regulation in *Lactococcus lactis***: a review. In: KONGO, M. (ed). Lactic Acid Bacteria - R& D for Food, Health and Livestock Purposes. InTechOpen. vol I. 467 – 486. 2013.

ALVES DE OLIVEIRA, R. et al. **Challenges and opportunities in lactic acid bioprocess design –** From economic to production aspects. Biochem Eng J. 133: 219 – 239. 2018.

AMORIM, H. V.; BASSO, L. C.; LOPES, M. L. **Evolution of ethanol production in Brazil**. In: BRYCE, J.H.; STEWARD, G.G. (ed). Distilled Spirits – Tradition and Innovation. Nottingham, UK: Nottingham University Press. 1: 143 – 148. 2004.

AMORIM, H. V. et al. **Scientific challenges of bioethanol production in Brazil**. Appl Microbiol Biotechnol. 91(5): 1267 – 1275. 2011.

ARIF, S. et al., **Physiochemical characteristcs nutritional properties and health benefits of sugarcane juice**. Non-alcoholic Beverages. Elsevier. 227 – 257. 2019.

AXELSSON, L. **Lactic Acid Bacteria: classification and physiology**. In: SALMINEN, S.; VON WRIGHT, A.; OUWEHAND, A.C. (eds). Latic acid bacteria: microbiology and functional aspects. Ed 3. New York: Marcel Dekker. p 1 – 66. 2004.

BASÍLIO, A. C. M. et al. **Detection and identification of wild yeast contaminants of the industrial fuel ethanol fermentation process**. Curr Microbiol. 56: 322 – 326. 2008.

BARABOTE, R. D.; SAIER, M. H. JR. **Comparative genomic analyses of the bacterial phosphotransferase system**. Microbiol Mol Biol Rev. 69: 608 – 634. 2005.

BARRE, P. **Identification of Thermobacteria and Homofermentative, Thermophilic, Pentose-utilizing *Lactobacilli* from High Termperature Fermenting Grape Musts**. Journal of Applied Bacteriology. 44: 125 – 129. 1978.

BASSO, L. C. et al. **Yeast selection for fuel ethanol production in Brazil**. FEMS Yeast Res. 8: 1155 – 1163. 2008.

BASSO, L. C.; BASSO, T. O.; ROCHA, S. N. **Ethanol production in Brazil:** The industrial process and its impact on yeast fermentation. In: BERNARDES, M. A. S. (ed). Biofuel production – Recent developments and prospects. InTechOpen. Vol 1530. 85 – 100. 2011.

BASSO, T. O. et al. **Homo- and heterofermentative lactobacilli differently affect sugarcane-based fuel ethanol fermentation**. Antonie van Leeuwenhoek.105:169–177. 2014.

BASSO, T. O.; LINO, F. S. O. **Clash of Kingdoms:** How Do Bacterial Contaminants Thrive in and Interact with Yeasts during Ethanol Production? In: BASSO, T.P.; BASSO, L.C. (eds). Fuel Ethanol Production from Sugarcane. IntechOpen. 23 – 37. 2018.

BATTESTI, A.; MAJDALANI, N.; GOTTESMAN, S. **The RpoS-Mediated General Stress Response in *Escherichia coli***. Annu Rev Microbiol. 65: 189 – 213. 2011.

BECKNER, M.; IVEY, M. L.; PHISTER, T. G. **Microbial contamination of fuel ethanol fermentations**. Lett Appl Microbiol. 53: 387 – 394. 2011.

BLANCATO, V. S.; MAGNI, C.; LOLKEMA, J. S. **Functional characterization and Me^{2+} ion specificity of a Ca^{2+}-citrate transporter from *Enterococcus faecalis***. FEBS Journal. 273: 5121 – 5130. 2006.

BOULTON, R. B. et al. **Principles and practices of winemaking**. New York: Chapman & Hall, 1996.

BOUTTE, C. C.; CROSSON, S. **Bacterial lifestyle shapes stringent response activation**. Trends in Microbiol. 21: 174 – 80. 2013.

CARR, F. J.; CHILL, D.; MAIDA, N. **The lactic acid bacteria: a literature survey**. Critical Reviews in Microbiology. 28: 281 – 370. 2002.

CASTRO, R. et al. **Characterization of the individual glucose uptake systems of *Lactococcus lactis*: mannose-PTS, cellobiose-PTS and the novel GlcU permease**. Molecular Microbiology. 71: 795–806. 2009.

CASTRO, R. E. N. et al. **Assessment of Sugarcane-Based Ethanol Production**. In: BASSO, T.P.; BASSO, L.C. (eds). Fuel Ethanol Production from Sugarcane. IntechOpen. 3 – 21. 2018.

CHANG, I. S. et al. **Bacterial contamination and its effect on ethanol fermentation**. J. Microbiol. Biotechnol. 5: 309 – 314. 1995.

CHENOL, E.; MACIÁN, M. C.; AZNAR, R. **Identification of *Carnobacterium*, *Lactobacillus*, *Leuconostoc* and *Pediococcus* by rDNA-based techniques**. Syst Appl Microbiol. 26(4): 546 – 556. 2003.

CIANI, M.; MACCARELLI, F.; FATICHENTI, F. **Growth and fermentation behaviour of *Brettanomyces/Dekkera* yeasts under different conditions of aerobics**. World Journal of Microbiology and Biotechnology. 19: 419 – 422. 2003.

CLAESSON, M. J. et al. **Multireplicon genome architecture of *Lactobacillus salivarius*.** Proceedings of the National Academy of Sciences. 103(17): 6718 – 6723. 2006.

COMASIO, A. et al. **The addition of citrate stimulates the production of acetoin and diacetyl by a citrate-positive *Lactobacillus crustorum* strain during wheat sourdough fermentation**. Int J Food Microbiol. 289: 88 – 105. 2019.

DA SILVA, P. K. N. et al. **Nutritional requirement for *Lactobacillus vini* growth in sugarcane derivative substrate of ethanol fermentation**. FEMS Microbiology Letters. 366(16): fnz202. 2019.

DE BARROS PITA, W. et al. **The ability to use nitrate confers advantage to *Dekkera bruxellensis* over *S. Cerevisiae* and can explain its adaptation to industrial fermentation processes**. Antonie Van Leeuwenhoek. 100: 99 – 107. 2011.

DE BARROS PITA, W. et al. **The influence of nitrate on the physiology of the yeast *Dekkera bruxellensis* grown under oxygen limitation**. Yeast. 30 (3):111 – 7. 2013.

DE OLIVA-NETO, P.; YOKOYA F. **Evaluation of bacterial contamination in a fed-batch alcoholic fermentation process**. World J Microbiol Biotechnol. 10: 967 – 999. 1994.

DE SOUZA, R. B. et al. **The consequences of *Lactobacillus vini* and *Dekkera bruxellensis* as contaminants of the sugarcane-based ethanol fermentation**. J Ind Microbiol Biotechnol. 39(11): 1645 – 1650. 2012.

DE SOUZA, R. B. et al. **Mineral Composition of the Sugarcane Juice and Its Influence on the Ethanol Fermentation**. Appl Biochem Biotechnol. 175: 209 – 222. 2015.

DUAR, R. M; et al. **Lifestyles in transition**: evolution and natural history of the genus *Lactobacillus*. FEMS Microbiol Rev. 41: S27 – 48. 2017.

ETHAYATHULLA, A. S. et al. **Struture-based mechanism for Na^+ / melibiose symport by MelB**. Nat Comun. 5: 3009. 2014.

GAROFALO, C. et al. T**he occurrence of beer spoilage lactic acid bacteria in craft beer production**. J Food. 80: M2845 – 2852. 2015.

GEIGER, T.; WOLZ, C. **Intersection of the stringent response and the CodY regulon in low GC Gram-positive bacteria**. International Journal of Medical Microbiology. 304(2): 150 – 5. 2014.

GOLDSTEIN, E. J. C.; TYRRELL, K. L.; CITRON, D. M. ***Lactobacillus* Species:** taxonomic complexity and controversial susceptibilities. Clinical Infectious Diseases: an Official Publication of the Infectious Diseases Society of America. 60(2): S98 – S107. 2015.

HAMMES, W. P.; VOGEL, R. F. **The genus *Lactobacillus***. In: WOOD, B.J.B.; HOLZAPFEL, W.H. (eds). The Genera of Lactic Acid Bacteria. London: Blackie Academic & Professional. v. 2, p 19 – 54. 1995.

HAMMES, W. P.; HERTEL, C. **The Genera *Lactobacillus* and *Carnobacterium***. In: DWORKING, M. et al. (eds) The Prokaryotes: Bacteria: *Firmicutes, Cyanobacteria.* New York: Springer. v. 4, p 320 – 403. 2006.

HAMMES W.; HERTEL C. **Genus I. *Lactobacillus Beijerink* 1901, 212^{AL}**. In: DE-VOS, P. et al. (eds). Bergey's manual of systematic bacteriology. 2 Ed.. Berlin: Springer. p 465 – 511. 2009.

HICKEY .M.; HILLIER, A.; JAGO, G. **Metabolism of Pyruvate and Citrate in Lactobacilli**. Aust J Biol Sci. **36**: 487 – 496. 1983.

HOLZAPFEL, W. H.; WOOD, B. J. B. **Introduction to the LAB**. In: HOLZAPFEL, W. H.; WOOD, B. J. B. (eds) . Lactic Acid Bacteria: biodiversity and taxonomy. Wiley-Blackwell. p 1 – 12. 2014.

HUGENHOLTZ, J. **Citrate metabolism in lactic acid bacteria**. FEMS Microbiol Rev. 12: 165 – 78. 1993.

INGLEDEW, W. M. **The biochemistry of alcohol production**. In: LYONS, T.P.; KELSALL, D.R.; MURTAGH, J.E. (eds). The alcohol textbook. Nottingham: Nottingham University Press. p 55 – 79. 1995.

INSKANDAR, C. F. et al. **Review of lactose and galactose metabolism in Lactic Acid Bacteria dedicated to expert genomic annotation**. Trends in Food Science & Technology. 121 – 132. 2019.

JAHREIS, K. et al. **Ins and outs of glucose transport systems in eubacteria**. FEMS Microbiological Review. 32: 891-907. 2008.

KANDLER, O. **Carbohydrate metabolism in lactic acid bacteria**. Antonie van Leeuwenhoek. 49: 209 – 224. 1983.

KANDLER, O.; WEISS, N. ***Lactobacillus***. In: SNEATH, P. H. A. et al. (eds). Bergey's manual of systematic bacteriology. Baltimore: Williams and Wilkins. 2: 103 – 121. 1986.

KASAI, K. et al. **Physiological analysis of the stringent response elicited in an extreme thermophilic bacterium, *Thermus thermophilus***. J Bacteriol. 188(20): 7111 – 7122. 2006.

KIM, B. H.; GADD, G. M. **Bacterial physiology and metabolism**. Cambridge: Cambridge University Press. 2008.

KONIGS, W. N. et al. **Lactic acid bacteria: the bug of the new millennium**. Curr Opin Microbiol. 3(3): 276 – 82. 2000.

KÖNIG, H.; FRÖHLICH, J. **Lactic Acid Bacteria**. In: KÖNIG, H.; UNDEN, G.; FRÖHLICH, J. (eds). Biology of Microorganisms on Grapes, in Must and Wine. Berlim Heidelberg: Springer. p 3 – 29. 2009.

KORITHOSKI, B.; KRASTEL, K.; CVITKOVITCH, D. G. **Transport and metabolism of citrate by *Streptococcus mutans***. J Bacteriol. 187(3): 4451 – 4456. 2005.

KOWALCZYK, M. et al. **Updates on Metabolism in Lactic Acid Bacteria in Light of "Omic" Technologies**. In: MOZZI, F.; RAYA, R.R; VIGNOLO. G.M. (eds) Biotechnology of Lactic Acid Bacteria: Novel Applications, 2nd Ed. West Sussex: Wiley-Blackwell. p 1 – 24. 2016.

LAËTITIA, G.; PASCAL, D.; YANN, D. **The citrate metabolism in homo- and heterofermentative LAB**: a selective means of becoming dominant over other microorganisms in complex ecosystems. Food and Nutr Sci. 5: 953 – 969. 2014.

LEITE, F. C. et al. **Quantitative aerobic physiology of the yeast *Dekkera bruxellensis*, a major contaminant in bioethanol production plants**. FEMS Yeast Research. 13(1): 34 – 43. 2013.

LIBERAL, A. T. S. et al. **Identification of the yeast *Dekkera bruxellensis* as major contaminant in continuous fuel ethanol fermentation**. J Appl Microbiol. 102(2): 538 – 547. 2007.

LIU, W. et al. **Biodiversity of Lactic Acid Bacteria**. In: Lactic Acid Bacteria: fundamental and practice. Zhang, H.; Cai, Y. (Eds) Dordrecht: Springer Science. p 103 – 203. 2014.

LONVAUD-FUNEL, A. **Lactic Acid Bacteria and Malolactic Fermentation in Wine**. Biotechnology of Lactic Acid Bacteria. Chichester, UK: John Wiley & Sons, Ltd. 231 – 47. 2015.

LORCA, G. et al. **Lactic Acid Bacteria**: Comparative Genomic Analyses of Transport Systems. In: MOZZI, F.; RAYA, R.R; VIGNOLO. G.M. (eds). Biotechnology of Lactic Acid Bacteria: Novel Applications. 1st Ed. West Sussex: Wiley-Blackwellp 73 – 87. 2010.

LUCENA, B. T. L. et al. **Diversity of Lactic Acid Bacteria of the bioethanol process**. BMC Microbiol. p 10: 298. 2010.

LUCENA, B. T. L. et al. **Genome Sequences of the Ethanol-Tolerant *Lactobacillus vini* Strains LMG 23202T and JP7.8.9**. J Bacteriol. p 194(11): 3018. 2012

MADIGAN, M. T. et al. **Microbiologia de Brock**. 14.ed. Porto Alegre: Artmed. 2016.

MAGNI, C. et al. **Mechanism of citrate metabolism in *Lactococcus lactis*: resistance against lactate toxicity at low pH**. J Bacteriol. 181(5): 1451 – 1457. 1999.

MAN, J. C.; ROGOSA, M.; SHARPE, M. E. **A medium for the cultivation of Lactobacilli**. J Appl Bacteriol. 23: 130 – 135. 1960.

MAYO, B. et al. **Updates in the metabolism of lactic acid bacteria**. In: Biotechnology of Lactic Acid Bacteria: Novel Applications, 1st Ed. Mozzi, F.; Raya, R.R; Vignolo. G.M. (Eds) West Sussex: Wiley-Blackwell. p 3 – 33. 2010.

MENDONÇA, A. A. et al. **First identification of Tn916-like element in industrial strains of *Lactobacillus vini* that spread the tet-M resistance gene**. FEMS Microbiol Lett. 363(3): fnv240. 2016.

MENDONÇA, A.A. **Aspectos fisiológicos e genéticos da bactéria *Lactobacillus vini* em condições de estresse.** 2018. 185 f. Tese (Doutorado) – Curso de Pós-graduação em Genética, Departamento de Genética, Universidade Federal de Pernambuco, Recife, 2012.

MENDONÇA, A. A. et al. ***Lactobacillus vini:* mechanistic response to stress by medium acidification**. Microbiology. 165(1): 26 – 36. 2019.

MONEDERO, V. et al. **The Phosphotransferase System of *Lactobacillus casei*:** Regulation of Carbon Metabolism and Connection to Cold Shock Response. Journal of Molecular Microbiology and Biotechnology. 12: 20-32. 2007.

MORITA, H. M.; et al. **Comparative genome analysis of *Lactobacillus reuteri* and *Lactobacillus fermentum* reveal a genomic island for reuterin and cobalamin production**. DNA Research. 15(3): 151–161. 2008.

MOZZI, F. **Lactic Acid Bacteria**. In: Encyclopedia of Food and Health. Caballero, B.; Finglas, P.M.; Toldrá, F. (Eds). Elsevier. p. 501 – 508. 2016.

MUTHAIYAN, A.; LIMAYEM, A.; RICKE, S. C. **Antimicrobial strategies for limiting bacterial contaminants in fuel bioethanol fermentations**. Progress in Energy and Combustion Science. 37(3): 351 – 370. 2011.

NARENDRANATH, N. V. et al. **Effects of lactobacilli on yeast-catalyzed ethanol fermentation**. Applied and Environmental Microbiology. 63(11): 4158 – 4163. 1997.

NARENDRANATH, N. V.; THOMAS, K. C.; INGLEDEW, W. M. **Effects of acetic acid and lactic acid on the growth of *Saccharomyces cerevisiae* in a minimal medium**. J Ind Microbiol Biotechnol. 26(3): 171 – 7. 2001.

NARENDRANATH, N. V. **Bacterial contamination and control in ethanol production**. In: In: The Alcohol Textbook: A reference for the beverage, fuel and industrial alcohol industries, 4th Ed. Jacques, K.A.; Lyons, T.P.; Kelsall, D.R. (Eds) Nottinham: Nottingham University Press. p 287 – 298. 2003.

NEUBAUER, H. et al. **Mechanism of maltose uptake and glucose excretion in *Lactobacillus sanfrancisco***. Journal of Bacteriology. 176(10): 3007 – 3012. 1994.

NEVES, A. R. et al. **Overview on sugar metabolism and its control in *Lactococcus lactis* – The input from in vivo NMR**. FEMS Microbiol Rev. 29(3): 531–554. 2005.

O'CALLAGHAN, J.; O'TOOLE, P. W. ***Lactobacillus*: Host-Microbe Relatioships**. Current Topics in Microbiology and Immunology. 358: 119 – 154. 2013.

PARENTE, D. C. et al. **Production of sensory compounds by means of the yeast *Dekkera bruxellensis* in different nitrogen sources with the prospect of producing cachaça**. Yeast. 31(1): 77 – 87. 2014.

PASSOTH, V.; BLOMQVIST, J.; SCHUNÜRER, J. ***Dekkera bruxellensis* and *Lactobacillus vini* Form a Stable Ethanol-Producing Consortium in a Commercial Alcohol Production Process**. Applied Environmental Microbiology. 73(13): 4354 – 4356. 2007.

PESSIONE, A.; LAMBERTI, C.; PESSIONE, E. **Proteomics as a tool for studying energy metabolism in lactic acid bacteria**. Mol Biosyst. 6(8): 1419 – 1430. 2010.

PEREIRA, L. P. et al. **The Physiological characteristics of the yeast *Dekkera bruxellensis* in fully fermentative conditions with cell recycling and in mixed cultures with *Saccharomyces cerevisiae***. Antonie van Leeuwenhoek. 101(3): 529 – 539. 2011.

POSTMA, P. W.; LENGELER, J. W.; JACOBSON, G. R. **Phosphoenolpyruvate: carbohydrate phosphotransferase systems of bacteria**. Microbiol Rev. 57(3): 543 – 594. 1993.

QUINTANS, N. G. et al. C**itrate metabolism and aroma compound production in lactic acid bacteria**. In: MAYO. B.; LÓPEZ, P.; PÉREZ-MARTÍNEZ, G. (eds). **Molecular Aspects of Lactic Acid Bacteria for Traditional and New Applications**. Vol 661. Kerala. 65–88. 2008.

REID, G. **The scientific basis for probiotic strains of *Lactobacillus***. Applied Environmental Microbiology. 65(9): 3763 – 3766. 1999.

RODAS, A. M., FERRER, S., PARDO, I. **Polyphasic study of wine *Lactobacillus* strains**: taxonomic implications. Int J Syst Evol Microbiol. 55(Pt 1): 197 – 207. 2005.

RODAS, A. M. et al. ***Lactobacillus vini sp. Nov.,* a wine lactic acid bacterium homofermentative for pentoses**. Int J Syst Evol Microbiol. 56(Pt 3): 513 – 517. 2006.

SALVETTI, E.; TORRIANI, S.; FELIS, G. E. **The genus *Lactobacillus***: a taxonomic update. Probiotics & Antimicro. Prot. 4: 217 – 226. 2012.

SCHELL, D. J. et al. **Contaminant occurrence, identification and control in a pilot-scale corn fiber to ethanol conversion process**. Bioresource Technology. 98: 2942 – 2948. 2007.

SCHLEIFER, K. H.; STACKEBRANDT, E. **Molecular systematics of prokaryotes**. Ann. Rev. Microbiol. 37: 143 – 187. 1983.

SEKIROV, I.; RUSSELL, S. L.; ANTUNES, C. M.; FINLAY, B. B. **Gut Microbiota in Healt Desease**. Physiol Rev. 90(3): 859 – 904. 2010.

SERRA, G.E. et al. **Contaminação da fermentação alcoólica “floculação do fermento”**. Brasil Açucareiro. 93: 26 – 31. 1979.

SILVA-FILHO, E.A. et al. **Yeast population dynamics of industrial fuel-ethanol fermentation process assessed by PCR-fingerprinting**. Antonie van Leeuwenhoek. 88: 13 – 23. 2005a.

SILVA-FILHO, E.A. et al. **Isolation by genetic and physiological characteristics of fuel-ethanol fermentative *Saccharomyces cerevisiae* strain with potencial for genetic manipulation**. J Ind Microbiol Biotechnol. 32(10): 481 – 486. 2005b.

SIRAGUSA, S. et al. **Fermentation and proteome profiles of *Lactobacillus plantarum* strains during growth under food-like conditions**. Journal of Proteomics. 366 – 380. 2014.

SONENSHEIN, A.L. ***CodY*****, a global regulator of stationary phase and virulence in Gram-positive bacteria**. Current Opinion in Microbiology. 8: 203 – 207. 2005.

SKINNER, K.A.; LEATHERS, T.D. **Bacterial contaminants of fuel ethanol production**. J Ind Microbiol Biotechnol. 31: 401 – 408. 2004.

SLOVER, C.; DANZIGER, L. ***Lactobacillus***: a Review. Clinical Microbiology Newsletter. 30(4): 23 – 27. 2008.

SMID, E.J.; KLEEREBEZEM, M. **Production of Aroma Compounds in Lactic Fermentations**. Annu Rev Food Sci Technol. 5: 313 – 26. 2014.

SUN, Z. et al. **Phylogenesis and Evolution of Lactic Acid Bacteria**. In: ZHANG, H.; CAI, Y. (eds). Lactic Acid Bacteria: fundamental and practice. Dordrecht: Springer Science. p 1 – 101. 2014.

THANH. V. N.; MAI, L. T.; TUAN, D. A. **Microbial diversity of traditional Vietnamese alcohol fermentation starters (banh men) as determined by PCR-mediated DGGE**. Int J Food Microbiol. 128: 268 – 73. 2008.

TIUKOVA, I.; EBERHARD, T.; PASSOTH, V. **Interaction of *Lactobacillus vini* with the ethanol-producing yeasts *Dekkera bruxellensis* and *Saccharomyces cerevisiae***. Biotechnol Appl Biochem. 61: 40 – 4. 2014.

TRAXLER, M.F. et al. **The global, ppGpp-mediated stringent response to amino acid starvation in *Escherichia coli***. Mol Microbioly. 68(5): 1128 – 48. 2008.

VENEMA, G.; KOKL, J.; VAN SINDEREN, D. **From DNA sequence to application**: possibilities and complications. Antonie van Leeuwenhoek. 76: 3 – 23. 1999.

VON WRIGHT, A.; AXELSSON, L. **Lactic Acid Bacteria**: an introdution. In: LAHTINEN, S. et al. (ed). Lactic Acid Bacteria – Microbiological and Functional Aspects 4th Ed. Boca Raton: CRC Press (Taylor & Francis Group). p 1 – 16. 2012.

WALKER, G.M. **Metals in Yeast Fermentation Processes**. Adv Appl Microbiol. p 197 – 229. 2003.

WANG, Y.; TASHIRO, Y.; SONOMOTO, K**. Fermentative production from renewable materials**: Recent achievements, prospects, and limits. J. Biosci. Bioeng. 119(1): 10 – 18. 2015.

WU, R.; LU, J. **Proteomics of Lactic Acid Bacteria** In: Lactic Acid Bacteria: fundamental and practice. Zhang, H.; Cai, Y. (Eds) Dordrecht: Springer Science. p 249 – 301. 2014.

YOKOYA, F.; DE OLIVA-NETO, P. Caracteristicas da floculação de leveduras por *Lactobacillus fermentum.* Rev de Microbiol. 22(1): 12 – 16. 1991.

ZABED, H. et al. **Bioethanol production from renewable sources:** Current perspectives and technological progress. Renewable and Sustainable Energy Reviews. 71: 475 – 501. 2017.

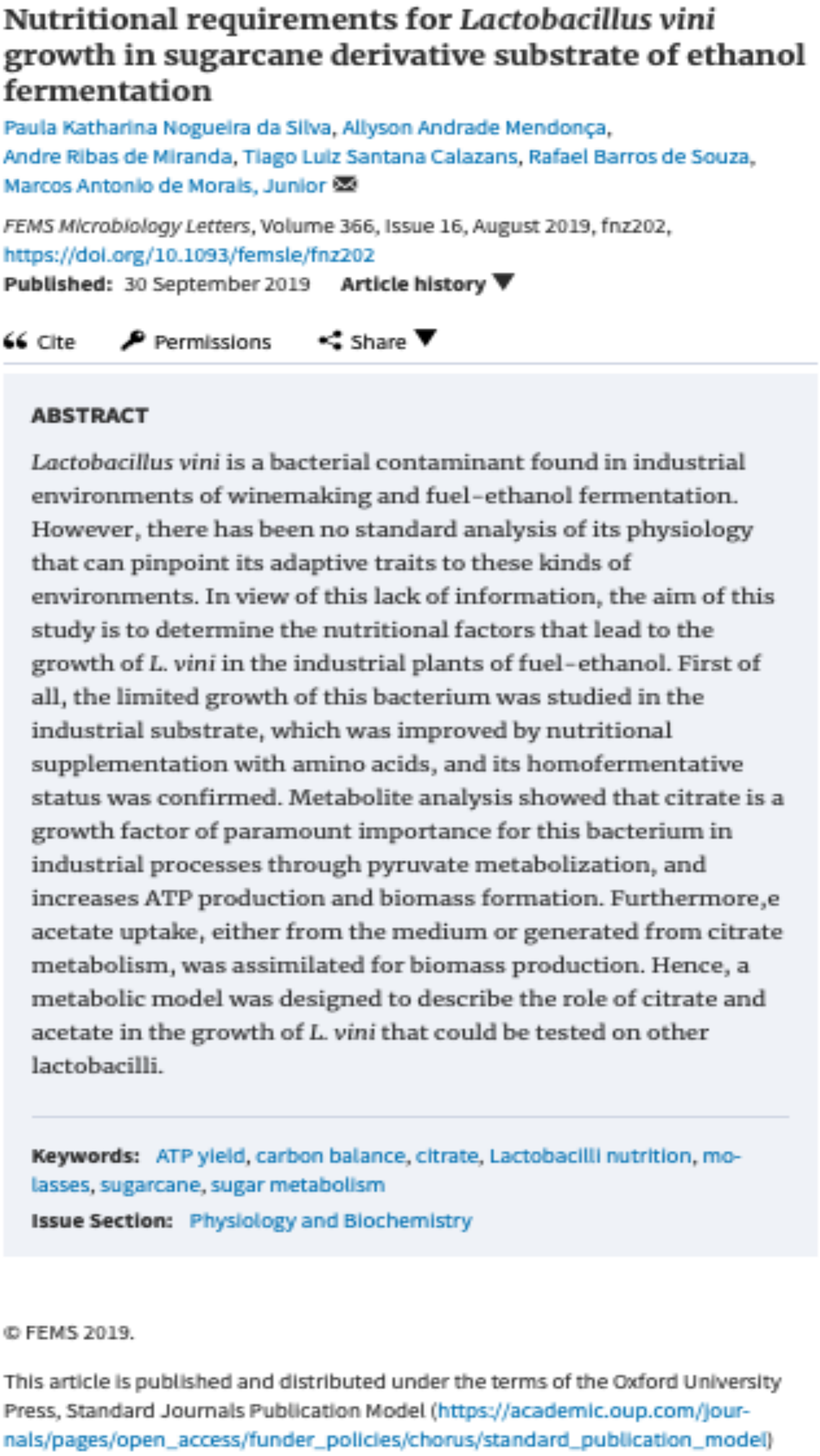
ZHANG, W. et al. **Comparative genome analysis of probiotic *Lactobacillus casei Zhang*.** In: Genomics II: bacteria, viruses and metabolic pathways. 1st ed. Hong Kong: iConcept Press Ltd. p. 276 – 296. 2013.

ZHANG, Y. et al. **Metabolic flux analysis of carbon balance in *Lactobacillus strains*.** Biotechnol Prog. 32(6): 1397 – 14036. 2016.

ZHAO, X. Q. et al. **Impact of zinc supplementation on the improvement of ethanol tolerance and yield of selfflocculating yeast in continuous ethanol fermentation. Journal of Biotechnology**. 139: 55–60. 2009.

ZHAO, X. Q.; BAI, F. W. **Mechanisms of yeast stress tolerance and its manipulation for efficient fuel ethanol production**. J Biotechnol. 144: 23 – 30. 2009.

APÊNDICE A – ARTIGO PUBLICADO NA REVISTA FEMS MICROBIOLOGY LETTERS



Nutritional requirements for *Lactobacillus vini* growth in sugarcane derivative substrate of ethanol fermentation

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ABSTRACT

Lactobacillus vini is a bacterial contaminant found in industrial environments of winemaking and fuel-ethanol fermentation. However, there has been no standard analysis of its physiology that can pinpoint its adaptive traits to these kinds of environments. In view of this lack of information, the aim of this study is to determine the nutritional factors that lead to the growth of *L. vini* in the industrial plants of fuel-ethanol. First of all, the limited growth of this bacterium was studied in the industrial substrate, which was improved by nutritional supplementation with amino acids, and its homofermentative status was confirmed. Metabolite analysis showed that citrate is a growth factor of paramount importance for this bacterium in industrial processes through pyruvate metabolization, and increases ATP production and biomass formation. Furthermore,e acetate uptake, either from the medium or generated from citrate metabolism, was assimilated for biomass production. Hence, a metabolic model was designed to describe the role of citrate and acetate in the growth of *L. vini* that could be tested on other lactobacilli.

Keywords: ATP yield, carbon balance, citrate, Lactobacilli nutrition, molasses, sugarcane, sugar metabolism

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Introduction

The diversity of lactic acid bacteria (LAB) in fermentation processes has been described in different environments, such as (i) the cornstarch-based ethanol production in the United States, (ii) the fermentation of barley wort in craft beer production and (iii) the fermentation of grape wort in wine production, as well as (iv) the ethanol production industry in Vietnam (Skinner and Leathers 2004; Rodas, Ferrer and Pardo 2005; Schell *et al.* 2007; Thanh, Mai and Tuan 2008; Garofalo *et al.* 2015; Lonvaud-Funel 2015; Duar *et al.* 2017). Fuel ethanol distilleries in Brazil use either sugarcane juice or sugarcane molasses, or sometimes a mixture of the two, as a substrate for fermentation. A number of yeasts, *Saccharomyces cerevisiae* and non-*S. cerevisiae* (Basílio *et al.* 2008), and homo- or heterofermentative bacteria (Lucena *et al.* 2010; Basso *et al.* 2014) are present in different cell counts in this ethanol fermentation process. Those most commonly found include *Lactobacillus fermentum*, *L. brevis*, *L. plantarum* and *Weissella paramesenteroides*. However, the *L. vini* species has attracted a good deal of attention on account of its high frequency and abundance among the LAB species in the distilleries of North- East Brazil (Lucena *et al.* 2010).

Although it was only described as a species in 2006, *L. vini* was first found in winemaking in 1978, when it was called the Mont 4 strain (Rodas *et al.* 2006). Later, *L. vini* was also identified as a contaminant in the ethanol production industry in Sweden and Brazil (Passoth, Blomqvist and Schnürer 2007; Lucena *et al.* 2010). These studies discovered that *L.*

vini can be harmful to ethanol production by (i) competing for the sugar with the main yeast, *S. cerevisiae*; (ii) producing lactic acid that can be toxic for *S. cerevisiae*; and (iii) through its presence somehow inducing the formation of flocs with *D. bruxellensis* and stimulating the fermentative metabolism of this yeast, while impairing the recycling of the yeast biomass (Passoth, Blomqvist and Schnürer 2007; de Souza *et al.* 2012; Tiukova, Eberhard and Passoth 2014).

The *L. vini* strain (described above) was classified as facultative anaerobic and homofermentative for hexose and pentose, since it only produces lactate as an end product (Rodas *et al.* 2006). Moreover, it can metabolize cellobiose, maltose, trehalose, sucrose, D-glucose, D-fructose, D-mannose and L-arabinose, but is not able to make use of glycerol, D-xylose, L-xylose, mannitol and lactose (Rodas *et al.* 2006). However, it should be noted that there is a wide metabolic variability among the LABs, especially the Lactobacillus group, even within the same species (Sun *et al.* 2014). This variation can be observed by the number of phosphotransferase systems (PTS) whose variability can be explained by the environmental pressure on substrate activity with a high concentration of different types of carbohydrates (Aleksandrzak-Piekarczyk 2013; Zhang *et al.* 2013). Moreover, LABs can be classified as either heterofermentative or homofermentative, depending on the type of metabolic pathway used to produce lactate, acetate and/or ethanol/CO_2, such as the glycolytic pathway, phosphate-pentose pathway and phosphoketolase pathway (Kandler 1983). Hence, this limited amount of information does not allow a comprehensive analysis to be conducted of the metabolism of *L. vini*, especially with regard to its ability to adapt to industrial environments such as those where sugarcane derivatives are used as substrates. Recent studies have reported the tolerance of *L. vini* to several forms of stress found in the fermentation process, such as acidification by organic and inorganic acids (Mendonça *et al.* 2019). In this regard, we showed

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that *L. vini* can take advantage of the substrate composition whenever some amino acids are present, which helps to keep the internal pH unaltered under acidic stress (Mendonça *et al.* 2019). Thus, some mechanisms for adapting to these environments have begun to be revealed.

Sugarcane juice is a quite simple substrate in terms of its main components when compared with barley or wheat wort, although its micro-composition can vary. Apart from the energy value of the high sugar content, several organic substances are found in the different sources of the cane, such as vitamins, phosphatides and amino acids (reviewed by Arif *et al.* 2019). This last group of components can be found in up to 2.5% (w/v) of the juice (Arif *et al.* 2019) and might be a key factor for bacterial acid tolerance, as stated above. The mineral composition is caused by the genetic diversity of the cane and soil quality (de Souza *et al.* 2015; Arif *et al.* 2019), and affects its fermentability when in a high concentration (de Souza *et al.* 2015).

The aim of this work is to study the metabolism of *Lactobacillus vini* JP7.8.9, a strain that is isolated from industrial ethanol fermentation (Lucena *et al.* 2010), in order to explain its high adaptability to the fermentative environment, especially in response to the nutritional composition of sugarcane juice. Hence, it was hoped the study would reveal the essential features of the *L. vini* metabolism that have practical repercussions on its behaviour in industrial fermentation processes.

Material and Methods

Bacteria strain and culture media

The strain of LAB used in this work was *Lactobacillus vini* JP7.8.9, which was isolated in the fuel-ethanol distillery of Japungu, located in Paraiba State, Brazil (Lucena *et al.* 2010). MRS broth (Difco Co., USA. Cat. Nº 288130) was used for cell cultivation, henceforth designated as commercial MRS. Solid MRS was prepared by adding bacto-agar to 2% (w/v). Sugarcane juice

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and molasses (as industrial substrates) were provided by distilleries at Japungu and Miriri (Santa Rita, PB). Supplementation of these substrates included the addition of individual components of the MRS compound: peptone (10 g L^{-1}), yeast extract (5 g L^{-1}), ammonium citrate (2 g L^{-1}) or a mix of vitamins (D-biotin 0.05 g L^{-1}, pantothenic acid 1 g L^{-1}, nicotinic acid 1 g L^{-1}, myoinositol 25 g L^{-1}, thiamine hydrochloride 1 g L^{-1}, pyrodoxal hydrochloride 1 g L^{-1} and p-aminobenzoic acid 0.2 g L^{-1}). All the medium components were purchased from Sigma-Aldrich Co. (St. Louis, USA). All the media liquid used was sterilised by filtration in advance, using 0.22 µm membrane filters. Solid medium was sterilised by autoclavation (at 121°C for 15 minutes). Alternatively, yeast extract made by the lysis of the industrial yeast *Saccharomyces cerevisiae* JP1, was used to supplement the industrial media and mimic industrial conditions. The yeast cells were cultured in YPD medium (yeast extract 10 g L^{-1}, peptone 20 g L^{-1}, dextrose 20 g L^{-1}) for this procedure, up to the stationary phase and collected by centrifugation in 50 mL conical tubes at 5000 rpm for five minutes at room temperature. The wet biomass was lysed by the addition of liquid nitrogen and glass beads (475-600 µm in diameter), followed by vortex shaking. The lysate was filtered in a membrane of 0.22 µm (Durapore PVDF. Merck Co., Cat nº P1438) and mixed with a small amount of the industrial media. An equivalent of 5g of the cell lysate was added to each litre of substrate to mimic the presence of dead yeast cell contents in the industrial environment.

Growth assay with sugarcane juice and molasses

L. vini cells collected from one colony of MRS plates were cultured in MRS broth for 16 h at 37°C without shaking. Cells from the cultures were used to inoculate MRS broth (as the reference medium) and to the industrial media of cane juice or molasses, with or without the addition of the supplements, initially to 0.1 absorbance units (A_{600nm}) in the total volume of 1 ml. Sugarcane juice and molasses were centrifuged at 5000 rpm for 15 minutes to remove the

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suspended solids, and both were diluted with distilled water to 100 g L^{-1} of total sugar. *Lactobacillus vini* cultivations were performed in FlowerPlates sealed in permeable film with BiolectorNA® microfermentation equipment (m2p-labs, Germany) at 32 °C and at constant humidity of 85% and speed of 800 rpm. Growth experiments were carried out in anaerobiosis with ultrapure nitrogen injection at 0.5 vvm. The laser readings of a range of suspended solids were carried out every 20 minutes and the light scattering values were transformed into optical density from the calibration factor defined in the laboratory for the suspension of *L. vini* cells. No culture medium pH variation was observed.

Analysis of sugar assimilation and glucose concentration

The MRS medium was prepared in-house from its components listed by the manufacturer (Difco) to test variations in glucose concentration and carbon source. Four different carbon sources were analysed: glucose, fructose, sucrose and maltose. The concentration of all the carbon sources was 20 g L^{-1}. The influence of sugar concentration on bacterial growth was tested by using glucose at 20, 40, 60, 80, 100 and 120 g L^{-1}. Cells pre-cultured in MRS medium were used for these growth assays to inoculate the MRS-specific media at initial concentration of 0.1 OD_{600nm} in sterile 96-well microplates to a final volume of 150 µL and grown in Synergy HT microincubator apparatus (BioTek, Swissland) at 37 °C, under high agitation. Absorbance readings were taken every twenty minutes. No culture medium pH variation was observed.

Growth experiments of varying citrate concentration

MRS medium was reproduced in the laboratory from its components with varying concentrations of citrate in the form of ammonium salt. The reference condition was MRS medium containing citrate concentration present in commercial MRS (8.8 mM). In a medium lacking citrate, it was replaced by sulphate (8.8 mM) as ammonium salt. The combined effect of glucose and citrate was tested by mixing both nutrients at 2, 20 or 200 mM each. Sugarcane

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juice and molasses were supplemented with citrate at 8.8 mM. During the cultivations, the cells were pre-cultured in commercial MRS medium and used to inoculate MRS-specific media at baseline of 0.1 OD_{600nm} in a total volume of 1 ml in FlowerPlates. The plates were incubated in BiolectorNA® microfermentor as previously described. The temperature was changed to 37 °C for optimal growth. No culture medium pH variation was observed.

Growth and fermentation experiment with glucose, citrate and pyruvate

Four conditions were tested for in-house made MRS: (i) with glucose (20 g L^{-1}) and ammonium citrate (8.8 mM citrate); (ii) with glucose (20 g L^{-1}) but without ammonium citrate; (iii) without glucose and with ammonium citrate (88 mM); (iv) with glucose (20 g L^{-1}) and sodium pyruvate (8.8 mM pyruvate). As described above, pre-cultured cells were re-suspended and inoculated into the MRS-specific media at initial concentration of 0.1 OD_{600nm} in 45 ml volume in flasks at 37 °C under static conditions. Absorbance readings were performed at intervals of 20, 24 and 48h, and at 24 and 48h samples were centrifuged and the supernatant was reserved for analysis of sugar consumption and the production of metabolites according to the protocol standards described below. All the fermentations were performed in triplicate, and the results obtained were represented as a simple mean average (± standard deviation). No culture medium pH variation was observed.

Metabolic analyses

The residual sugar concentration and metabolites produced were determined by high pressure liquid chromatography (HPLC) in Agilent Technologies 1200 Series equipment with the model HPX87H column (BioRad) in mobile phase of sulfuric acid to 5 mM at 0.6 mL min^{-1} and 45 °C. The metabolites were identified by means of a refractive index detector with the aid of a standards solution through the methodology described (Zhao and Bai 2009) with adaptations (de Souza *et al.* 2012).

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Results and Discussion

Growth analysis of *Lactobacillus vini* in industrial media

Growth experiments were carried out that used sugarcane juice or molasses as an industrial substrate, and laboratory medium MRS as the reference condition. The results showed a slower growth of *L. vini* strain JP7.8.9 in the industrial substrates than was the case with MRS (Fig. 1). Hence, the composition of industrial substrates lack any of the essential factors that inhibit bacterial growth, despite high counts of its presence in many industrial samples of ethanol fermentation (Lucena *et al.* 2010). In view of this paradox, we investigated the nutritional limitations of the sugarcane juice and molasses that impair bacterial growth and the factors that enable *L. vini* growth in such poor substrates. The first condition examined was the fact that the concentration of sugar in the industrial substrates (120 to 140 g L^{-1}) was higher than in MRS (20 g L^{-1}). The variation in glucose concentration from 20 to 120 g L^{-1} had absolutely no effect on the growth pattern of *L. vini* JP7.8.9 (Fig. 2A) which meant that the excess of sugar in the industrial substrate was not the cause of the low growth of the bacterium. In addition, sugarcane juice and molasses contain sucrose together with their hydrolysis products of glucose and fructose (Arif *et al.* 2019). There was no significant difference in growth when the glucose was replaced with sucrose or fructose, which suggests that the composition of sugars was also not related to the growth deficiency in the industrial media (Fig. 2B). Maltose, an α-disaccharide of glucose mainly found in beer wort, was used as an example of a different industrial substrate. Growth experiments showed that maltose was less efficiently used than the other sugars tested (Fig. 2B), and thus that sugar composition could be a determinant for this deficiency in another industrial setting, but not for sugarcane derivatives.

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After ruling out the effect of sugar on growth efficiency, we turned our attention to other components of the MRS medium. In this case, industrial substrates were supplemented with peptone, yeast extract and/or a mixture of vitamins. The addition of peptone with or without vitamins or addition of yeast extract alone equally increased bacterial growth in sugarcane juice to the same extent (Fig. 3A) or sugarcane molasses (Fig. 3B), although the final biomass only reached 55% and 40% of that obtained with MRS medium, respectively. This suggested that the fastidious nature of *L. vini*, which is a characteristic of lactobacilli (Duar *et al.* 2017), did not allow its growth in substrates that are low in amino acids, the common nutrients in both supplements. In view of this, the reported presence of *L. vini* in the industrial substrates might be because the bacterial growth was assisted by some sort of supplementation within these compounds.

One possible source of these kinds of nutrients could be the intracellular contents of dead cells of the microbial population during the fermentation cycles. To test this hypothesis, we supplemented the substrates with a lysate of *S. cerevisiae* industrial strain JP1 in an attempt to mimic the industrial conditions in which the yeast cells are dying. In this case, bacterial growth in lysate-supplemented juice was twice as high as in non-supplemented juice (Fig. 3A), which is evidence of the positive effects of the intracellular content of yeast on bacterial growth. However, this was still at a much lower level than that observed when commercial yeast extract was added (Fig. 3A). It should be noted that this experiment used the estimate of 10% of cell lysis that was calculated for the yeast population in each fermentation cycle (Basso, Basso and Rocha 2011). It resulted in a supplementation of the substrate that was close to 0.1% (w/v) with intracellular content. This concentration is ten times lower than the amount used with commercial yeast extract, which might explain such a difference in growth. Our previous results showed that the count of *L. vini* remained stable when co-cultivated with

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the yeast *S. cerevisiae* and *D. bruxellensis* in sugarcane, - a condition in which the yeast cells remain intact (de Souza *et al.* 2012). It is nevertheless reported that there was a greater presence of *L. vini* in the fermentation when the industrial substrate was treated with pasteurisation which might lead to the lysis of the microbial autochthonous population of cane juice (de Souza *et al.* 2012). Thus, the data provided here should indeed reflect the industrial conditions, where the bacterial population growth is limited and does not surpass the yeast population.

However, this supplementation did not promote growth in sugarcane molasses (Fig. 3B). This can be explained by the composition of this substrate which contains harmful compounds, such as oxidized molecules produced during the sugar milling, and the high content of the minerals and metals that are concentrated by evaporation during the sugar production. It would require a much higher content of this material released by dead yeasts to allow the growth of *L. vini* in this substrate, as was observed when commercial yeast extract was added to molasses (Fig. 3B). It also explains the absence of *L. vini* in most of the industrial plants that use molasses as a fermentation substrate, which it is not the case for other lactobacilli (Lucena *et al.* 2010).

Influence of citrate on *L. vini* growth patterns

As well as peptone and yeast extract, MRS contains citrate in the form of ammonium salt, which is widely described in the literature as supporting lactobacilli growth (Laëtitia, Pascal and Yann 2014; Kowalczyk *et al.* 2015) and industrial processes (Comasio *et al.* 2019). The role of this six-carbon organic acid in bacterial growth was tested by replacing ammonium citrate (AC) with ammonium sulphate (AS) in MRS composition, while keeping the equimolar quantities of ammonium ion. The absence of citrate extended the lag growth phase (Fig. 4A). After this prolonged non-growth period, the cultures in citrate-lacking MRS started to grow

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slowly and after 36 h reached the same final biomass of the cultures in citrate-containing MRS (data not shown). This result proved that citrate is important to fasten growth of *L. vini*, but is not indispensable for its long-term growth. Afterwards, the effect of different citrate concentrations in the final biomass was tested. The results showed that at least 2.5 mM of this substrate was necessary to boost the bacterial growth and concentrations above 7.5 mM had a slightly further increment in this parameter (Fig. 4B). Hence, sugarcane juice was supplemented with citrate to the same concentration found in the original composition of MRS (8.8 mM). Sugarcane juice only supported 15% of the final bacterial growth with regard to MRS (Fig. 4C). The addition of citrate to sugarcane juice doubled the bacterial growth, and reached 30% of the reference condition, while the presence of peptone or yeast extract supported bacterial growth to 50%. A clear additional effect of citrate was observed for substrates supplemented with either peptone or yeast extract, and increased bacterial growth until it reached 90% of the reference condition (Fig. 4C). This means that, as well as the bacterial requirements for amino acids and/or oligopeptides present in peptone or in the yeast extract, citrate is an important growth factor for this bacterium in the industrial substrates.

Finally, an assessment was made to determine whether citrate can also be used as a sole carbon source or in glucose-limited condition. No growth was detected at the initial glucose concentration of 2 mM and a slight growth was observed at 20 mM glucose, irrespective of the presence of citrate (data not shown). The results showed that citrate is not being used by the bacterial cells as the sole carbon source. Additionally, no bacterial growth was observed at 200 mM citrate, whatever glucose concentration was present in the medium. This concentration is close to that used as a food preservative owing to its toxic effects on most of the bacterial cells, which also seems to be the case for *L. vini*. Among the combinations tested, optimal bacterial growth was achieved at 200 mM glucose and 20 mM citrate (Fig. 4D).

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It should be further evaluated if the increase in growth obtained when citrate was at 20 mM, offsets the increase of the cost of this nutrient in concentrations above 7.5 mM. Altogether, these results found that citrate is mainly used as a supplementary nutritional source in a medium containing enough glucose to provide the carbon needed for *L. vini* growth. There is an abundance of citrate in nature and it is regarded as an important energy source for the growth of different bacteria found in food matrices (Laëtitia, Pascal and Yann 2014).

The effect of citrate on the *L. vini* metabolism

It has been theoretically conjectured that lactobacilli metabolize citrate (6C) through its initial breakdown to acetate (2C) and oxaloacetate (4C) by means of citrate lyase, and that the oxaloacetate is further decarboxylated to pyruvate (3C) (Hugenholtz 1993; Kowalczyk *et al.* 2015). Hence, pyruvate could become the product of citrate metabolization that is related to energy production. Since this route would be redox neutral, pyruvate cannot be reduced to lactate and must thus be deviated to the production of acetyl-CoA. As well as this, acetyl-CoA is further converted to acetyl-P using inorganic phosphate. This energy-rich molecule is the substrate for ATP production that results in acetate (Laëtitia, Pascal and Yann 2014), which can be either a) expelled to the medium, b) converted to acetoin or diacetyl by the bacterial cells (Hugenholtz 1993), or c) further assimilated into the biomass. In view of this possibility, we replaced citrate with its metabolization product (pyruvate) at a concentration of 8.8 mM in MRS. The results showed that both citrate and pyruvate resulted in the same final biomass at 24 h of cultivation (Table 1), which proves that pyruvate indeed metabolically replaces citrate. This finding provides experimental evidence that corroborates the use of metabolic models proposed for other lactobacilli (Hickey, Hillier and Jago 1983; Quintans *et al.* 2008). Hence, it can be concluded that pyruvate is ultimately responsible for the increment in the growth of *L. vini* in the industrial substrates supplemented with citrate (Fig. 4).

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Final concentrations of metabolic products were measured from cultures of *L. vini* in different medium compositions after 48 h of cultivation (Table 1). Regardless of the presence of citrate or pyruvate, the cells completely consumed the glucose and produced lactate at about 70% of the maximum theoretical efficiency as a single fermentation product (Table 1) and as a result, confirmed the homofermentative status given to *L. vini* (Rodas *et al.* 2006). This high conversion efficiency shows the potential of *L. vini* for the industrial production of lactate, an important bioproduct with many applications for the synthesis of biodegradable polymers (Alves de Oliveira *et al.* 2018). When citrate or pyruvate were added to the medium, the final biomass increased by about 30% relative to the medium without citrate (Table 1). As a result, the carbon from citrate or pyruvate is assimilated for cell formation and is not dissimilated to a fermentation product such as CO_2, acetate of ethanol. It also led to the conclusion that the acetate that is first produced by the breakdown of citrate and ultimately formed from pyruvate, is assimilated into the biomass.

As well as citrate, acetate is also present in MRS at an initial concentration of 61 mmol/L and our findings showed that this metabolite was differentially consumed by the bacterial cells in the different media: 33 mmol consumed in MRS+glucose, 24 mmol consumed in MRS+glucose+pyruvate and 15 mM consumed in MRS+glucose+citrate (Table 1). Hence, less external acetate was required when citrate was co-consumed with glucose. In the case of MRS+glucose+citrate, the bacterial cells consumed 9 mmol less acetate from the medium than in MRS+glucose+pyruvate. This is in accordance with the calculation that the breakdown of 8.8 mmol citrate present in MRS first releases 8.8 mmol acetate and then 8.8 mmol of oxaloacetate, which ultimately releases a further 8.8 mmol acetate from pyruvate. This results in 17.6 mmol acetate available for biomass formation and was very close to the experimental difference of 16 mmol between the MRS medium with or without citrate (Table 1). If pyruvate

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was used in the medium, 9 mmol more acetate was experimentally consumed by the cells than the citrate medium (Table 1). This is once again very close to the hypothesis that there is a metabolic deficit of 8.8 mmol acetate when pyruvate is metabolised. From these data, it was possible to devise a metabolic model, which is closely in line with that previously proposed (Laëtitia, Pascal and Yann 2014), and suggests that citrate is metabolised to generate ATP, as well as to ultimately produce acetate that is used together with the external acetate for biomass formation (Fig. 5). Pyruvate is thus able to fulfil the citrate requirement for ATP production, although it imposes the need for more acetate uptake from the medium (Fig. 5). This uptake is even higher in the absence of citrate or extracellular pyruvate (Fig. 5).

Acetate is the building block for lipid biosynthesis in reactions that use NADPH as an electron donor. This reduced coenzyme is produced in the pentose phosphate (PPP) pathway from glucose. The data in Table 1 showed that 70% of glucose is converted to lactate, while suggesting that 33.6 mmol of glucose (30%) deviated to PPP to generate 67.2 mmol NADPH. This amount is theoretically enough to convert 34 mmol acetate to lipids, which was the exact amount calculated from the consumption of acetate (Table 1). The bacterial cells grow slowly when the biosynthesis of lipid depends entirely on the influx of acetate, in the absence of citrate (Fig. 4A), and takes a longer incubation period to reach the same final biomass obtained in complete MRS. In the light of this, it can be concluded that the rate of growth of *L. vini* JP7.8.9 is limited by the rate of acetate uptake. The stimulating effect of citrate on the growth of lactobacilli was pointed out in the original description of the MRS medium (Man, Rogosa and Sharpe 1960). Since then, no explanation was given for the use of citrate until the recent metabolic model postulated that this nutrient played a role in the growth of lactobacilli (Laëtitia, Pascal and Yann 2014). Hence, this study is the first to provide consistent

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experimental data that at least explain this nutritional requirement for *L. vini* and assist in elucidating the behaviour of this bacterium in industrial fermentation processes.

Conclusion

This study has shown that the limited growth of *L. vini* in sugarcane derivative substrates is the consequence of a lack of oligopeptides/amino acids and citrate as key nutritional requirements. This limitation can be overcome by the lysis of the part of the microbial population, (mainly yeast cells), that release these supplements in the substrate and allow bacterial cells to grow during the fermentation process. It also consolidates the metabolization of citrate through pyruvate, by providing enough acetate for biomass formation. This information on *L. vini* metabolic features reveals significant aspects of its performance in industrial fermentation processes.

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Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest: The author declares no conflict of interest

References

Aleksandrzak-Piekarczyk T. Lactose and β-Glucosides Metabolism and Its Regulation in *Lactococcus lactis*: A Review. In: Kongo, J. Marcelino (University of the Azores P (ed.). *Lactic Acid Bacteria - R & D for Food, Health and Livestock Purposes*. Vol i. InTech, 2013, 467–86.

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bioprocess design—From economic to production aspects. *Biochem Eng J* 2018;**133**:219–39.

Arif S, Batool A, Nazir W *et al.* Physiochemical Characteristics Nutritional Properties and Health Benefits of Sugarcane Juice. *Non-Alcoholic Beverages*. Elsevier, 2019, 227–57.

Basílio ACM, de Araújo PRL, de Morais JOF *et al.* Detection and Identification of Wild Yeast Contaminants of the Industrial Fuel Ethanol Fermentation Process. *Curr Microbiol* 2008;**56**:322–6.

Basso LC, Basso TO, Rocha SN. Ethanol Production in Brazil: The Industrial Process and Its Impact on Yeast Fermentation. In: Bernandes MA dos S (ed.). *Biofuel Production-Recent Developments and Prospects*. Vol 1530. IntechOpen, 2011, 85–100.

Basso TO, Gomes FS, Lopes ML *et al.* Homo- and heterofermentative lactobacilli differently affect sugarcane-based fuel ethanol fermentation. *Antonie Van Leeuwenhoek* 2014;**105**:169–77.

Comasio A, Harth H, Weckx S *et al.* The addition of citrate stimulates the production of acetoin and diacetyl by a citrate-positive *Lactobacillus crustorum* strain during wheat sourdough fermentation. *Int J Food Microbiol* 2019;**289**:88–105.

Duar RM, Lin XB, Zheng J *et al.* Lifestyles in transition: evolution and natural history of the genus *Lactobacillus*. *FEMS Microbiol Rev* 2017;**41**:S27–48.

Garofalo C, Osimani A, Milanović V *et al.* The occurrence of beer spoilage lactic acid bacteria in craft beer production. *J Food Sci* 2015;**80**:M2845–52.

Hickey M, Hillier A, Jago G. Metabolism of Pyruvate and Citrate in Lactobacilli. *Aust J Biol Sci* 1983;**36**:487.

Hugenholtz J. Citrate metabolism in lactic acid bacteria. *FEMS Microbiol Rev* 1993;**12**:165–78.

Kandler O. Carbohydrate metabolism in lactic acid bacteria. *Antonie Van Leeuwenhoek*

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1983;**49**:209–24.

Kowalczyk M, Mayo B, Fernández M *et al.* Updates on metabolism in lactic acid bacteria in light of "Omic" technologies. In: Mozzi F, Raya RR, Vignolo GM (eds.). *Biotechnology of Lactic Acid Bacteria*. Chichester, UK: John Wiley & Sons, Ltd, 2015, 1–24.

Laëtitia G, Pascal D, Yann D. The citrate metabolism in homo- and heterofermentative LAB: A selective means of becoming dominant over other microorganisms in complex ecosystems. *Food Nutr Sci* 2014;**05**:953–69.

Lonvaud-Funel A. Lactic Acid Bacteria and Malolactic Fermentation in Wine. *Biotechnology of Lactic Acid Bacteria*. Chichester, UK: John Wiley & Sons, Ltd, 2015, 231–47.

Lucena BT, Dos Santos BM, Moreira JLS *et al.* Diversity of lactic acid bacteria of the bioethanol process. *BMC Microbiol* 2010;**10**:298.

Man JC de, Rogosa M, Sharpe ME. A medium for the cultivation of Lactobacilli. *J Appl Bacteriol* 1960;**23**:130–5.

Mendonça AA, da Silva PKN, Calazans TLS *et al. Lactobacillus vini*: Mechanistic response to stress by medium acidification. *Microbiol (United Kingdom)* 2019;**165**:26–36.

Passoth V, Blomqvist J, Schnürer J. *Dekkera bruxellensis* and *Lactobacillus vini* form a stable ethanol-producing consortium in a commercial alcohol production process. *Appl Environ Microbiol* 2007;**73**:4354–6.

Quintans NG, Blancato V, Repizo G *et al.* Citrate metabolism and aroma compound production in lactic acid bacteria. In: Mayo B, López P, Pérez-Martínez G (eds.). *Molecular Aspects of Lactic Acid Bacteria for Traditional and New Applications*. Vol 661. Kerala, 2008, 65–88.

Rodas AM, Chenoll E, Macián MC *et al. Lactobacillus vini* sp. nov., a wine lactic acid bacterium homofermentative for pentoses. *Int J Syst Evol Microbiol* 2006;**56**:513–7.

Rodas AM, Ferrer S, Pardo I. Polyphasic study of wine *Lactobacillus* strains: taxonomic

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implications. *Int J Syst Evol Microbiol* 2005;**55**:197–207.

Schell DJ, Dowe N, Ibsen KN *et al.* Contaminant occurrence, identification and control in a pilot-scale corn fiber to ethanol conversion process. *Bioresour Technol* 2007;**98**:2942–8.

Skinner K a, Leathers TD. Bacterial contaminants of fuel ethanol production. *J Ind Microbiol Biotechnol* 2004;**31**:401–8.

de Souza RB, de Menezes JAS, de Souza RFR *et al.* Mineral Composition of the Sugarcane Juice and Its Influence on the Ethanol Fermentation. *Appl Biochem Biotechnol* 2015;**175**, DOI: 10.1007/s12010-014-1258-7.

de Souza RB, Santos BM dos, de Fátima Rodrigues de Souza R *et al.* The consequences of *Lactobacillus vini* and *Dekkera bruxellensis* as contaminants of the sugarcane-based ethanol fermentation. *J Ind Microbiol Biotechnol* 2012;**39**:1645–50.

Sun Z, Yu J, Dan T *et al.* Phylogenesis and Evolution of Lactic Acid Bacteria. In: Zhang H, Cai Y (eds.). *Lactic Acid Bacteria*. Dordrecht: Springer Netherlands, 2014, 1–101.

Thanh VN, Mai LT, Tuan DA. Microbial diversity of traditional Vietnamese alcohol fermentation starters (banh men) as determined by PCR-mediated DGGE. *Int J Food Microbiol* 2008;**128**:268–73.

Tiukova I, Eberhard T, Passoth V. Interaction of *Lactobacillus vini* with the ethanol-producing yeasts *Dekkera bruxellensis* and *Saccharomyces cerevisiae*. *Biotechnol Appl Biochem* 2014;**61**:40–4.

Zhang W, Sun Z, Wu R *et al.* Comparative genome analysis of a new probiotic strain Lactobacillus casei Zhang. *Genomics II-Bacteria, Viruses and Metabolic Pathways*. 2013, 276–296.

Zhao XQ, Bai FW. Mechanisms of yeast stress tolerance and its manipulation for efficient fuel ethanol production. *J Biotechnol* 2009;**144**:23–30.

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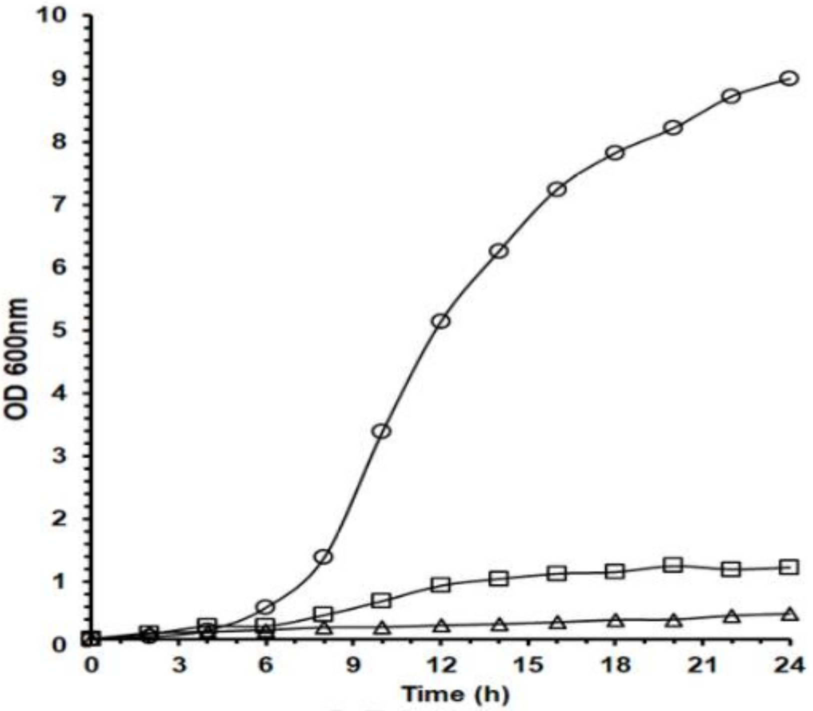

Figure 1. Growth curve of *Lactobacillus vini* JP7.8.9 in MRS medium containing glucose at 20 g L^{-1} (○) and in industrial media sugarcane juice (□) or molasses (△) containing total sugar at 100 g L^{-1}.

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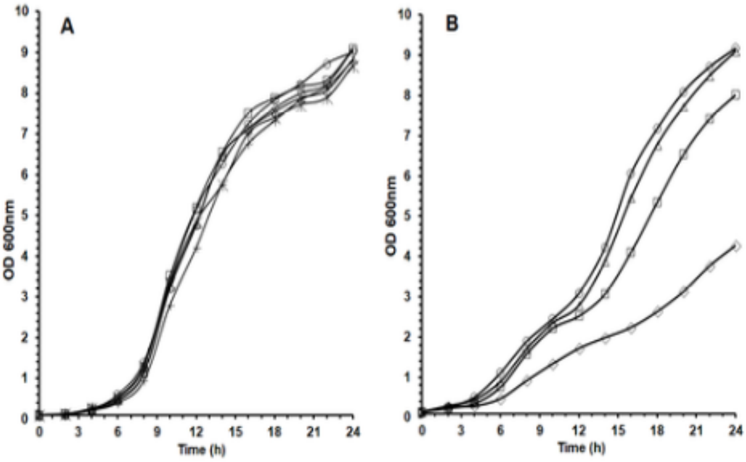

Figure 2. Effect of the sugar on the growth of *Lactobacillus vini* JP7.8.9 in MRS medium. (**A**) Effect of varying concentrations of glucose at 20 g L^{-1} (○), 40 g L^{-1} (□), 60 g L^{-1} (△), 80 g L^{-1} (◇), 100 g L^{-1} (★) and 120 g L^{-1} (+). (**B**) Effect of different sugars such as glucose (○), fructose (□), sucrose (△) or maltose (◇) at 20 g L^{-1}.

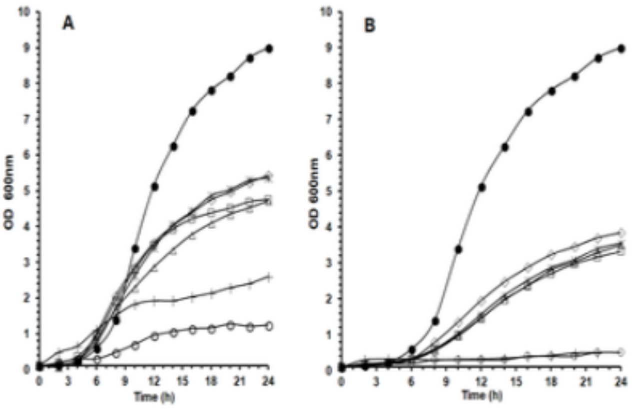

Figure 3. Effect of supplementation of industrial substrates such as sugarcane juice (○, panel **A**) or sugarcane molasses (○, panel **B**) on the anaerobic growth of *Lactobacillus vini* JP7.8.9 with yeast extract (□), peptone (△), yeast extract+peptone (◇), peptone+vitamins (★) or yeast lysate (+). MRS medium (●) was used as reference.

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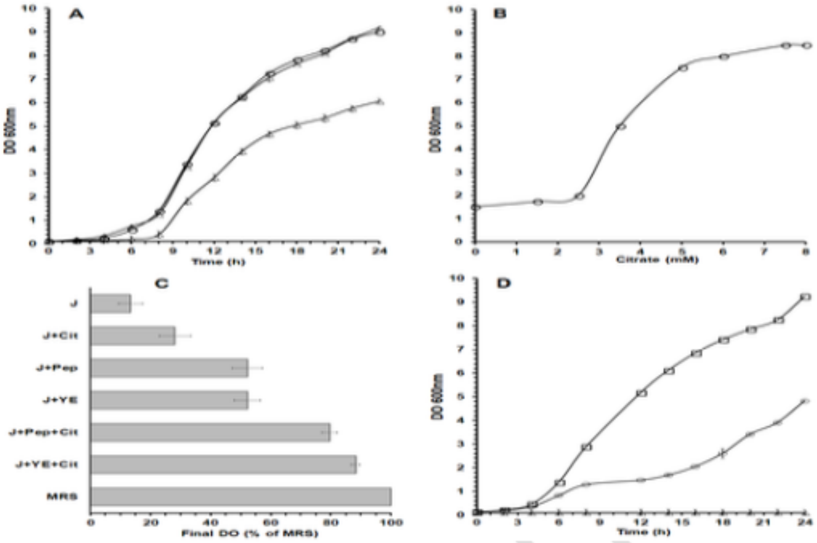

Figure 4. Influence of citrate on the growth of *Lactobacillus vini* JP7.8.9. (A) commercial MRS (○), MRS medium prepared in-house with ammonium citrate (□) and MRS prepared in-house with ammonium sulphate (△). (**B**) Final bacterial biomass achieved in MRS medium prepared in-house containing varying amounts of ammonium citrate in different concentrations. (**C**) Final bacterial biomass in sugarcane juice (J) with or without supplementation with yeast extract (YE), peptone (Pep), citrate (Cit) relative to complete MRS medium (100%). (**D**) Effect of varied concentrations of ammonium citrate at 2 mM (○), 20 mM (□) or 200 mM (△) on bacterial growth in MRS medium containing glucose at 20 mM. N.B. All citrate concentrations mentioned referred to the citrate anion, not to the salt form.

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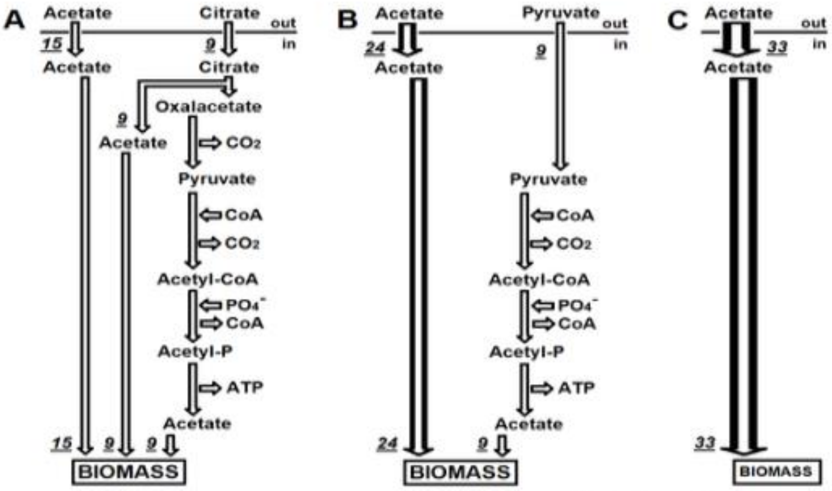
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Figure 5. Proposed metabolic pathways for the growth of *Lactobacillus vini* JP7.8.9 in medium containing acetate and citrate (panel A), acetate and pyruvate (panel B) or solely acetate (panel C). Metabolites in the medium (out) involves cellular uptake (in) and are used for biomass formation. The underlined numbers represent the amount of metabolite assimilated. The width of the arrow related to the assimilation of extracellular acetate represents the amount of this metabolite. The size of the source indicating biomass formation, represents the comparison of the final biomass in each growth condition.

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Table 1. Final concentrations of metabolites and biomass from cultivation of *Lactobacillus vini* JP7.8.9 for 48 h in MRS prepared in-house with different formulations[a].

Parameter	Glu (112 mM) Ace (61 mM)	Glu (112 mM) Ace (61 mM) Cit (8.8 mM)	Glu (112 mM) Ace (61 mM) Pyr (8.8 mM)
Glucose (mM)[b]	-112	-112	-112
Acetate (mM)[b]	-31 (±0.1)	-15.3 (±0.32)	-22.7 (±0.21)
Citrate (mM)[b]	n.a.[e]	-8.8	n.a.
Pyruvate (mM)[b]	n.a.	n.a.	-8.8
Biomass (OD_{600nm})[c]	6.06 (±0.047)	9.09 (±0.087)	9.08 (±0.086)
Lactate (mM)[c]	157.1 (±11.6)	165.8 (±13.6)	170.3 (±16.1)
Lactate yield (g g^{-1})[d]	0.70	0.74	0.76

[a] Glu: glucose; Ace: acetate; Cit: citrate; Pyr: pyruvate. [b] metabolite consumed. [c] metabolite produced. [d] lactate produced/glucose consumed. [e] not applicable.

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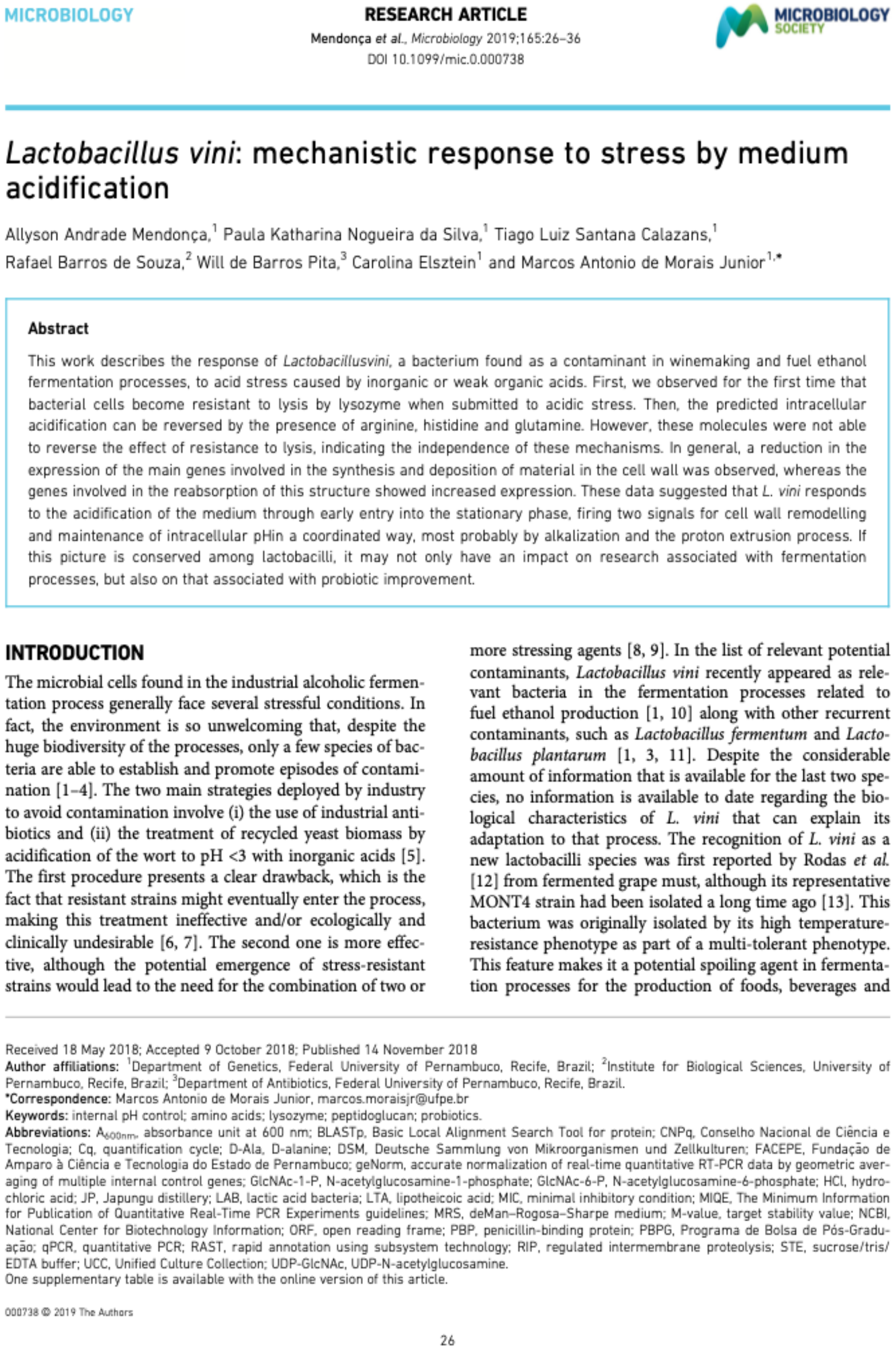
APÊNDICE B – ARTIGO PUBLICADO NA REVISTA MICROBIOLOGY



MICROBIOLOGY

RESEARCH ARTICLE
Mendonça *et al*., *Microbiology* 2019;165:26–36
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Lactobacillus vini: mechanistic response to stress by medium acidification

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Rafael Barros de Souza,[2] Will de Barros Pita,[3] Carolina Elsztein[1] and Marcos Antonio de Morais Junior[1,*]

Abstract

This work describes the response of *Lactobacillusvini*, a bacterium found as a contaminant in winemaking and fuel ethanol fermentation processes, to acid stress caused by inorganic or weak organic acids. First, we observed for the first time that bacterial cells become resistant to lysis by lysozyme when submitted to acidic stress. Then, the predicted intracellular acidification can be reversed by the presence of arginine, histidine and glutamine. However, these molecules were not able to reverse the effect of resistance to lysis, indicating the independence of these mechanisms. In general, a reduction in the expression of the main genes involved in the synthesis and deposition of material in the cell wall was observed, whereas the genes involved in the reabsorption of this structure showed increased expression. These data suggested that *L. vini* responds to the acidification of the medium through early entry into the stationary phase, firing two signals for cell wall remodelling and maintenance of intracellular pHin a coordinated way, most probably by alkalization and the proton extrusion process. If this picture is conserved among lactobacilli, it may not only have an impact on research associated with fermentation processes, but also on that associated with probiotic improvement.

INTRODUCTION

The microbial cells found in the industrial alcoholic fermentation process generally face several stressful conditions. In fact, the environment is so unwelcoming that, despite the huge biodiversity of the processes, only a few species of bacteria are able to establish and promote episodes of contamination [1–4]. The two main strategies deployed by industry to avoid contamination involve (i) the use of industrial antibiotics and (ii) the treatment of recycled yeast biomass by acidification of the wort to pH <3 with inorganic acids [5]. The first procedure presents a clear drawback, which is the fact that resistant strains might eventually enter the process, making this treatment ineffective and/or ecologically and clinically undesirable [6, 7]. The second one is more effective, although the potential emergence of stress-resistant strains would lead to the need for the combination of two or more stressing agents [8, 9]. In the list of relevant potential contaminants, *Lactobacillus vini* recently appeared as relevant bacteria in the fermentation processes related to fuel ethanol production [1, 10] along with other recurrent contaminants, such as *Lactobacillus fermentum* and *Lactobacillus plantarum* [1, 3, 11]. Despite the considerable amount of information that is available for the last two species, no information is available to date regarding the biological characteristics of *L. vini* that can explain its adaptation to that process. The recognition of *L. vini* as a new lactobacilli species was first reported by Rodas *et al.* [12] from fermented grape must, although its representative MONT4 strain had been isolated a long time ago [13]. This bacterium was originally isolated by its high temperature-resistance phenotype as part of a multi-tolerant phenotype. This feature makes it a potential spoiling agent in fermentation processes for the production of foods, beverages and

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Keywords: internal pH control; amino acids; lysozyme; peptidoglucan; probiotics.
Abbreviations: A_{600nm}, absorbance unit at 600 nm; BLASTp, Basic Local Alignment Search Tool for protein; CNPq, Conselho Nacional de Ciência e Tecnologia; Cq, quantification cycle; D-Ala, D-alanine; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen; FACEPE, Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco; geNorm, accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes; GlcNAc-1-P, N-acetylglucosamine-1-phosphate; GlcNAc-6-P, N-acetylglucosamine-6-phosphate; HCl, hydrochloric acid; JP, Japungu distillery; LAB, lactic acid bacteria; LTA, lipotheicoic acid; MIC, minimal inhibitory condition; MIQE, The Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines; MRS, deMan–Rogosa–Sharpe medium; M-value, target stability value; NCBI, National Center for Biotechnology Information; ORF, open reading frame; PBP, penicillin-binding protein; PBPG, Programa de Bolsa de Pós-Graduação; qPCR, quantitative PCR; RAST, rapid annotation using subsystem technology; RIP, regulated intermembrane proteolysis; STE, sucrose/tris/EDTA buffer; UCC, Unified Culture Collection; UDP-GlcNAc, UDP-N-acetylglucosamine.
One supplementary table is available with the online version of this article.

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fuel ethanol. However, no systematic studies have been performed to date to uncover the cellular and molecular mechanisms behind this tolerance. In this sense, the present work intends to initiate studies on the mechanisms of stress tolerance in this bacterium in the continuation of the definition of its genomic composition [14].

The cell wall of lactobacilli can be considered to be the first barrier of the cells against stressful environmental conditions. It is composed of a glycanpolymer chain of N-acetylglucosamine and N-acetylmuramic acid linked to each other by β1–4 bonds [15]. The glycan strands are cross-linked by short-stem peptides attached to each N-acetylmuramic acid molecule [16, 17]. The amino acid composition of the stem peptides may change among species [15, 16], but the last two D-Ala-D-Ala dipeptides are very conserved. This peptidoglycan structure is responsible for maintaining cell integrity, as well as its shape [18]. Long strands of glycerol-phosphate in the lipoteichoic acid (LTA) are attached to the cell membrane or are linked directly to the cell wall [16, 19]. LTA might often undergo insertions of D-ala into its chain, and this is performed by the proteins coded by the *dlt* operon. It comprises four genes coding for enzymes involved with the activation, transport and esterification of LTA with D-ala residues [20].

The way in which the components of peptidoglycan are modified when bacterial cells are challenged by stressing agents is critical for the maintenance of cell viability and its establishment in the environment [18]. In order to survive in stressful environments, the peptidoglycan might be chemically changed, which in turn, interferes in the normal cell wall metabolism, such as its sensitivity to autolysins [18, 21]. Those hydrolytic enzymes display muramidase lysozyme-like activity, which is required for the relaxation of peptidoglycan cross-linked network, allowing cell elongation and ultimately cell division [22]. These enzymes are found from prokaryotes to eukaryotes and conserve the biological activity of muramidase [23]. In mammals, these enzymes are part of the immune system that is responsible for killing pathogens [24]. On the other hand, the pathogens have developed mechanisms that lead to the resistance of the cell wall to degradation by mammalian lysozymes [24, 25].

In model organisms, such as *Bacillus subtilis*, the protective mechanism against cell wall hydrolysis by lysozyme involves the activation of modifiers, which acetylate the peptidoglycan components or esterify LTA [26]. These modifications are performed by (i) the enzyme encoded by the gene *oatA* and (ii) enzymes encoded by genes in the *dlt* operon, all of which are under the regulatory control of the σ^V transcription factor [26]. In an inactivated state, σ^V is linked to the anti-sigma factor RsiV in the inner face of the cell membrane. However, the presence of lysozyme activates the RIP system that releases σ^V from RsiV [27]. Free σ^V recruits RNA polymerase to target promoters of *oatA*, *pbpX* and the *dlt* operon [26]. The inactivation of the *dlt* operon in *L. plantarum* leads to an abnormal sensitivity to autolysin [28]. To date, only a few descriptions of the mechanism of resistance to lysozyme in lactobacilli are available, but there is already an indication that it is an inducible mechanism that is triggered by stressful medium conditions [29].

The list of adverse conditions that can damage the cell envelope in the industrial fermentation process includes a pH variation of the fermentation wort promoted by the yeast fermentative activity, or that may also be due to the production of weak organic acids (acetic and lactic) by the bacterial contaminant population itself [30, 31]. The use of inorganic acids, such as hydrochloric or sulphuric, in order to control microbial contamination [5] imposes a severe acid stress that can damage the cell envelope. In addition, variations in temperature, ethanol concentration and solute concentration (triggering osmotic stress) and the presence of oxidant molecules can also affect that cell component. Therefore, we aimed to study the major mechanisms used by *L. vini* cells to resist and tolerate the presence of stressing agents and conditions that occur naturally in or that are added to the processes for the control of bacterial population.

In the present work, we challenged bacterial cells against a set of stressing agents and evaluated how they affect the cell envelope structure, using resistance to lysozyme as an indicator for peptidoglycan network reorganization. The results showed that an inducible mechanism of resistance to lysozyme was only activated upon acid stress (caused by weak organic or strong inorganic acids) in such a way that it was independent from the mechanisms of internal pH maintenance. The genetic response as evaluated by the relative expression of key genes indicated that the mechanistic process behind this adaptation induced by acid stress is dependent on the reduction of peptidoglycan deposition and/or its stimulated reabsorption. The energetics of this process was also discussed. The results presented herein provide information to elucidate the mechanisms of acid resistance in lactic acid bacteria (LAB), which is relevant for (i) their use as a probiotic, facing the acid environment in the gastro-intestinal tract; (ii) their use as a dairy producer; and (iii) for improving strategies to combat the spoilage of food and ethanol fermentation processes.

METHODS

Strain, medium and minimum inhibitory condition (MIC) determination

L. vini JP 789 was isolated from the industrial ethanol fermentation process [1]. The genome of this strain, as well as the type strain DSM 20605, were sequenced [14] and are available in GenBank database (https://www.ncbi.nlm.nih.gov/genome/?term=lactobacillus+vini). Bacterial cells were maintained in de Man–Rogosa–Sharpe (MRS) broth (Merck) containing 2 % agar. Cultivations (seed cultures) were performed in MRS broth for 24 h at 37 °C without shaking. For the MIC assays, cells from seed cultures were used to inoculate 1 ml of MRS broth containing stressing agents at different concentrations for an initial cell density of 0.005 A_{600nm}. Then, 150 µl of each condition was transferred to wells in sterile microtitre plates. The stress agents were: (1) lactic acid (up to 200 mM, with a 10 mM gradient) and acetic acid (up to 400 mM, with a 25 mM gradient) as weak organic acids; (2) hydrochloric acid (from pH 8 to 2, with a 0.5 gradient) as inorganic acid; (3) hydrogen peroxide (up to 1.5 mM, with a 0.1 mM gradient) as oxidative stress; and (4) sodium chloride (up to 1 M, with a 0.1 M gradient) as osmotic stress. The pH of the MRS medium did not change with the addition of the stressing agents, except in the case of hydrochloric acid. Cultivations were performed for 48 h at 37 °C without shaking. For heat-shock assays, the cultures were prepared as above and cultivated for 48 h in a TS-100 thermoshaker (Biosan) at different temperatures. The MIC was defined as the first condition that completely inhibited cell growth, while sub-MIC corresponded to a condition that interfered with cell growth.

Growth kinetic analyses were performed by preparing microtitre plates as above and cultivating cells in a Sinergy HT multireader device (Biotek, Switzerland) at 37 °C with continuous shaking. Absorbance measurements were recorded automatically every 30 min for 48 h. The specific growth rate was calculated from the slope of the linear portion of the exponential growth phase. Cell viability at the MIC was evaluated by taking 10 µl of the cultures at 0, 24 and 48 h and dropping it onto MRS agar plates. Plates were incubated for 96 h in anaerobic jars with an Anaerocult A anaerobiosis generator (Merck). The acid protective effect of amino acids, as previously reported by Wu *et al.* [32], was also tested by supplementing MRS broth with each one of the 20 amino acids to 50 mM. Variation in the pH of the MRS was achieved by adjusting the initial pH to 3.5 after amino acid supplementation. Afterward, the media were filter-sterilized. All experiments were performed in biological duplicate with technical triplicates. Therefore, the numbers represent the mean value of six measurements (±SD) for each sample.

Cell lysis by lysozyme

Seed cells were diluted to 0.1 A_{600nm} in MRS containing stressing agents at sub-MIC, defined as 60 mM lactic acid, 150 mM acetic acid, 600 mM sodium chloride, 0.8 mM hydrogen peroxide or 316 µM H^+ (medium adjusted to a pH of 3.5 with HCl) and incubated for 60 min at 37 °C. For the heat-shock assay, seed cells diluted in MRS broth were incubated for 60 min 42 °C. As a positive control, seed cells were diluted in MRS and incubated without treatment. Afterward, the cells were collected by centrifugation and resuspended with one-tenth of the original volume in STE buffer (300 mM sucrose, 100 mM tris-HCl and 500 mM EDTA-NaOH, pH 8) containing lysozyme at 10 mg ml^{-1}. The buffered cell suspensions were transferred (150 µl) to microtitre plates and incubated at 37 °C. Variations in absorbance were recorded every 30 min for 24 h in a Sinergy HT multireader device. As negative control, untreated cells from the positive control were incubated without enzymes. The experiments were performed in biological duplicate with technical triplicates for all conditions. Therefore, the numbers represent the mean value of six measurements (±SD) for each sample.

ORF annotation, primer design and validation

The annotation of ORFs was performed in the automatic system RAST (http://rast.nmpdr.org) [33] with the available genome of the strain *L. vini* JP 789 [14]. An additional step to confirm the ORF annotation was made with the amino acid sequences of those ORFs compared with the NCBI database using the tool BLASTP (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins). The ORFs representative of σ^V regulon (σ^V, *dltD* and *pbpX*), peptidoglycan precursor biosynthesis (*glmS*, *glmU* and *murB*) and extra-cellular peptidoglycan biosynthesis (*pbp1A*-like ORFs) and D-alanine metabolism (*al-L*, *al-R* and *al-T*) were identified and the nucleotide sequences were collected for primer design. The genes *groES*, *fusA*, *rpoB*, *recA* and *pcrA* were candidates for the reference gene as previously described [34]. The reference genes were indicated by GeNorm analysis to be the pair with a target stability value (M-value) lower than 0.15. The homologues were identified in the *L. vini* genome and the nucleotide sequence was then collected for primer design.

The primers were designed with the online tool Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) with the parameters: amplicon size, 70–150 pb; primer size, 19–21 pb; minimum GC content of 20–80 %; primer melting temperature from 59 to 61 °C; maximum G or C in the last five bases in the 3′ end; and amplicon melting temperature ≥80 °C. Moreover, the maximum acceptable length for a mononucleotide repeat was 4. The primers that had been designed were additionally analysed with the online tool OligoAnalyser (https://www.idtdna.com/calc/analyzer). The complete list of primers is available in the supplementary material (Table S1, available in the online version of this article). The PCR reaction mix was made with 1× Sybr Green Master Mix (Promega, USA) and 0.2 µM of primer mix with a final volume of 10 µl. The reactions were performed in technical triplicates. The primer was considered to have been validated when the slope of the regression curve ranged from −3.1 to −3.5, as in previous studies [35–37].

RNA isolation, cDNA synthesis and gene expression

For RNA isolation, seed cells were suspended in 10 ml MRS to 1.0 A_{600nm} and treated with 60 mM lactic acid, 150 mM acetic acid or 316 µM H^+ (medium adjusted to a pH of 3.5 with HCl). After 1 h of static incubation at 37 °C, cells were collected by centrifugation and total RNA was extracted with a hot acid phenol protocol. Briefly, the cell sediment was resuspended in 500 µl of AE buffer (10 mM EDTA, 50 mM of sodium acetate, final pH 5.3). Then, volumes of 50 µl of SDS 10 % and 500 µl of equilibrated phenol (pure phenol adjusted to pH 5.3 with 50 mM sodium acetate solution) were sequentially added to the cell suspension. The mixture was mixed by vortex to homogenization and incubated for 10 min at 65 °C, with intermittent mixing by vortex every 2 min. After this time the tubes were chilled and kept on ice. An equal volume of phenol/chloroform solution (5 equilibrated phenol : 1 chloroform) was added and the mixtures were vortexed for 1 min. The tubes were submitted to centrifugation at 20 000 ***g*** at room temperature. The aqueous upper phase was collected to new tubes and mixed with an equal volume of pure chloroform, and then the procedure above was repeated. Finally, the upper phase was collected in new tubes and the RNA was purified with the illustra RNAspin Mini RNA Isolation kit (GE Healthcare) following the manufacturer's instructions. RNA integrity was evaluated directly on 1 % agarose gel prepared with TAE buffer (40 mM of tris, 10 mM of EDTA, 20 mM of acetic acid) and stained with 0.5 mg ml^{-1} of ethidium bromide, and its concentration was performed in Nanodrop (Thermo Fisher, USA). RNA samples were stored at −80 °C until use.

The cDNA was synthetized with the ImProm-II Reverse Transcription System (Promega, USA), following the manufacturer's instructions. Each reaction was performed with 0.5 µg of RNA in a final volume of 20 µl. RT negative controls were also prepared with the same amount of RNA diluted in water (same concentration as cDNA sample) in order to estimate contamination with genomic DNA. The samples were stored at −20 °C until use. The cDNA was used for qPCR reaction under the same conditions as described above for the primer validation in biological triplicates and technical triplicate. The Cq values of each replicate from reference genes and tested genes were used for normalization and relative expression quantification according to MIQE guidelines [38]. The results were submitted to analysis of variance (ANOVA) with the post hoc Tukey honestly significant difference (HSD) test (http://astatsa.com/OneWay_Anova_with_TukeyHSD/) ($P \leq 0.05$).

RESULTS AND DISCUSSION

Stress response-induced resistance to lysozyme

One can reasonably assume that the establishment of *L. vini* in the industrial process might be the consequence of its tolerance to the stressful conditions observed in that environment [1, 39], such as high concentrations of ethanol and organic acids, osmotic pressure and high temperature [31]. This assumption arises mainly because this species was originally isolated from wine production as temperature-resistant bacteria [12, 13]. To test the tolerance of *L. vini* JP 789, we performed several assays with increasing concentrations of several stressor agents. The results represented the MIC values that were defined for lactic acid, acetic acid, ethanol, medium pH, sodium chloride, hydrogen peroxide and heat-shock stress (Fig. 1a). However, none of those inhibitory conditions led to a significant loss of viability (Fig. 1a), meaning that we were dealing with bacteriostatic conditions. A decrease in the cell population viability was only observed after long-term exposure (48 h) to ethanol at the MIC (Fig. 1a). The intensity of each stress condition endured by the strain was greater than or at least equal to the reported values in the bioethanol industrial environment [31], meaning that this bacterium is not eliminated by any of these conditions if established individually in the fermentation process. This tolerance profile should contribute to the establishment of *L. vini* in the process, as has been reported in Brazil [1] and Sweden [39].

Additionally, we evaluated whether the stressor agents had any impact on the resistance of the cells to lysozyme after treatment with sub-MIC doses. The cell lysis profile was not changed by the incubation with ethanol, sodium chloride, hydrogen peroxide and heat-shock stress Fig. 1b, c). However, incubation with lactic, acetic or hydrochloric acid increases the resistance of the bacterial cell wall to lysis (Fig. 1c). Acid stress is the most frequent stressing condition faced by the LAB, since it is a self-imposed condition that arises from the production of organic acids [40]. In bioethanol industries, other sources of acid stress can be found, such as (i) the extrusion of H$^+$ by the fermenter yeast during fermentative activity, which makes a smaller contribution to the overall acidification, and (ii) the pre-treatment of yeast biomass for cell recycling using inorganic acid to control the contamination [41]. The acid treatment is employed before each fermentation cycle, which means that every 8 to 12 h cells have to face an acid treatment that drops the medium pH close to 2.0. This treatment is used repeatedly during the months of the fermentation season [31]. In spite of being the major stress condition studied for LAB, acid stress has never been reported as changing the resistance to lysozyme in any bacterial species. It seems, indeed, that the phenomenon might contribute to the increase of bacterial survival during the industrial acid treatment.

The induction of lysozyme resistance has been reported for *L. fermentum* after entry into the stationary phase [42] and for *L. casei* upon exposure to osmotic stress [29]. Unlike for other micro-organisms, entrance into the stationary phase in lactobacilli is influenced by the accumulation of organic acids, such as lactic acid [43, 44]. Therefore, we assumed that exposure to lactic acids would mimic the entry into the stationary phase in *L. vini* as well. To test this possibility, we submitted 48 h grown cultures of *L. vini*, which had already reached the stationary growth phase according to the growth kinetics of the control condition (Fig. 2a) and submitted them to the lysozyme test. The results showed that bacterial cells in the stationary phase were more resistant to lysozyme than freshly cultured cells (Fig. 1c). This indicated that *L. vini* behaves similarly to *L. fermentum*, by changing the envelope structure upon entry into the stationary phase, which makes it inaccessible to the action of lysozyme.

Amino acid catabolism influences lysozyme induction in an acidic environment

At this point, we showed that *L. vini* cells most likely respond to medium acidification by modifying the properties of their cell walls as the first barrier against the stressing condition. Afterwards, it is important to understand how

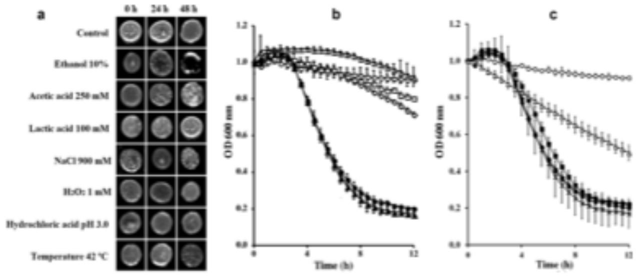

Fig. 1. Effect of different stressing agents on *Lactobacillus vini* cells. (a) Spot test for cell viability after the exposure of bacterial cells to the minimal inhibitory condition (MIC) for the stressing agents for 0, 24 and 48 h of incubation. (b) Susceptibility of *L. vini* cells to lysozyme after pre-treatment at sub-MIC with the stressor agents acetic acid (150 mM, △), lactic acid (60 mM, ◊), HCl (pH 3.0, □) or ethanol (8.5 %, ▲). (c) Susceptibility of *L. vini* cells to lysozyme after pre-treatment at sub-MIC with the stressor agents NaCl (600 mM, ■), hydrogen peroxide (0.8 mM, ♦) or temperature (40 °C, *). In both panels, the negative control refers to cells that were not submitted to lysozyme treatment (○), while lysis control refers to cells that weere not submitted to sub-MIC pre-treatment and then treated with lysozyme (•). The lysis profile of cells in the stationary phase of growth is shown (Δ).

L. vini cells deal with medium acidification and how they respond to such a stressful condition.

It is well-known that a drop in the medium pH leads to the acidification of the cell cytoplasm in the genus *Lactobacillus* [45]. A widespread mechanism to control internal pH in LAB employs reactions involving amino acids as substrates for the production of alkaline compounds or for the consumption of protons from the cytoplasm [40]. To test whether such a protective effect of amino acids is also present in *L. vini*, we performed growth experiments in the presence of sub-MIC inorganic acid (medium adjusted to pH 3.5 with HCl) in MRS supplemented with amino acids. Three out of 20 amino acids showed an acid-protective effect, improving growth in the acid medium (Fig. 2a) and restoring the specific growth rates to be close to the level of the control cultures (Fig. 2b). Arginine restored cell growth, although the final cell biomass was only 67 % of that of the control cultures. The arginine deiminase pathway is most likely the cause of the protective effect of arginine against acid stress, working by releasing ammonia, an alkali-like molecule that helps cells to maintain the internal pH [46, 47]. We identified the genes for this pathway in the genome of *L. vini* for both strains JP 789 and DSM 20605 (data not shown). Thus, we might assume that the arginine deiminase pathway is also present in *L. vini* and can

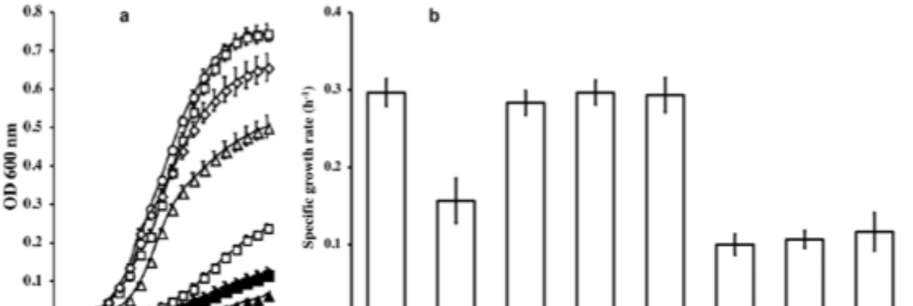

Fig. 2. Effect of amino acid supplementation on the growth of *Lactobacillus vini* in MRS medium adjusted to pH 3.5 with hydrochloric acid. (a) Profile of the growth kinetics of bacterial cells in acid MRS supplemented with arginine (△), glutamine (◊), histidine (□), asparagine (♦), methionine (▲) or glycine (■). The references were MRS medium not adjusted to acid pH (○) or pH-adjusted MRS without amino acid supplementation (•). (b) Specific growth rates calculated from the slope of the log portion of the growth curves shown in (a).

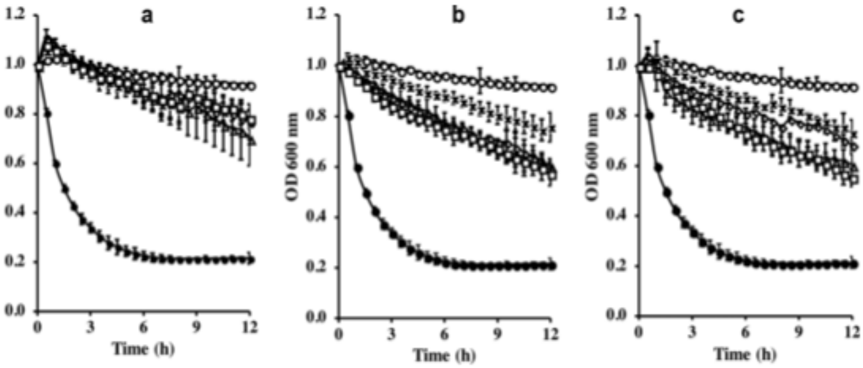

Fig. 3. Effect of the acid-protective amino acids on the profile of lysozyme resistance in *Lactobacillus vini*. Bacterial cells were incubated in MRS medium adjusted to sub-MIC pH (3.5) with HCl (a) in MRS containing sub-MIC lactic acid (60 mM) (b) or in MRS medium containing acetic acid (150 mM) (c). Each of these media were supplemented with arginine (△), glutamine (◊) or histidine (□). In each panel, the negative control refers to cells that were not submitted to lysozyme treatment (○), the lysis control refers to cells that were not submitted to sub-MIC pre-treatment and then treated with lysozyme (•), and the positive control refers to cells that were submitted to sub-MIC pre-treatment with each acid and then treated with lysozyme (*).

protect cells against acid stress. Glutamine also restored the growth rate to the control level, but the final biomass was only 87 % of that of the control condition. The catabolism of this amino acids goes through a glutamine lyase reaction that also releases ammonia [46, 47]. However, the gene encoding glutamine lyase was not found in the *L. vini* genome (data not shown). Finally, histidine showed the greatest protective effect by restoring both the growth rate and the final biomass to the control levels. This effect has been reported for other LAB, working by a decarboxylation H^+-consuming mechanism that produces histamine and CO_2 [48]. However, the gene that encodes histidine decarboxylase was also not found in *L. vini* genome (data not shown). So far, these are the only protective mechanisms to be described for glutamine and histidine, which indicates that alternative enzymes or new mechanisms should be operating in *L. vini.* Furthermore, we observed that the supplementation of MRS with methionine, glycine or asparagine had a synergistic negative effect with the acid stress, reducing the final biomass production (Fig. 2a) and growth rate (Fig. 2b). The mechanisms underlying this phenomenon are currently unknown and they are beyond the scope of the present work. The remaining 14 amino acids showed neither positive nor negative effects on growth and lysozyme resistance upon bacterial exposure to acid stress (data not shown).

At this point we showed that stress by acid (organic or inorganic) produced a bacteriostatic effect accompanied by an increase in cell resistance to lysozyme. Since the addition of histidine, glutamine or arginine reverted that deleterious effect, we tested whether these amino acids could also restore the lysozyme-sensitivity phenotype. The results showed that none of them were able to revert the cell lysis profile of acid-treated cells to the control level (Fig. 3), indicating that the control of cell growth and lysis resistance rely on different mechanisms.

Genetic response to acid stress in respect of the σ^V regulon response

In view of the proposed reorganization of the cell wall indicated by the induced resistance to lysozyme, we turned our attention to the expression of key genes in the biosynthesis and deposition of metabolites in this structure, as well as its remodelling (Fig. 4). The data presented above indicated that acid stress, induced by both weak organic and inorganic acid, might have important implications for the growth and maintenance of *L. vini* in industrial conditions, leading to changes in resistance to cell lysis. In addition to the physiological aspects reported above, we analysed the genes that could be involved in the acid stress response. Several genes that were recovered from the *L. vini* genome were identified by RAST annotation as being responsible for the structure and maintenance of cell envelope integrity, the first barrier against unfavourable environmental conditions. geNorm analysis indicated that the most stable genes between the treatments conditions were *rpoB* and *fusA*, with an M-value of 0.05. Therefore, these genes were used to normalize the expression of the tested genes.

The first set of genes included those of the $\boldsymbol{\sigma}^V$ regulon, which includes the $\boldsymbol{\sigma}^V$ gene itself. This gene encodes an extracellular $\boldsymbol{\sigma}^V$ transcription factor that interacts with RNA polymerase to transcribe genes required for resistance to lysozyme [26, 27]. Since pretreatment with HCl, lactic acid and acetic acid leads to lysozyme resistance (Fig. 1c), it was expected that there would be a direct co-relation with $\boldsymbol{\sigma}^V$ gene expression levels. However, a threefold up-regulation

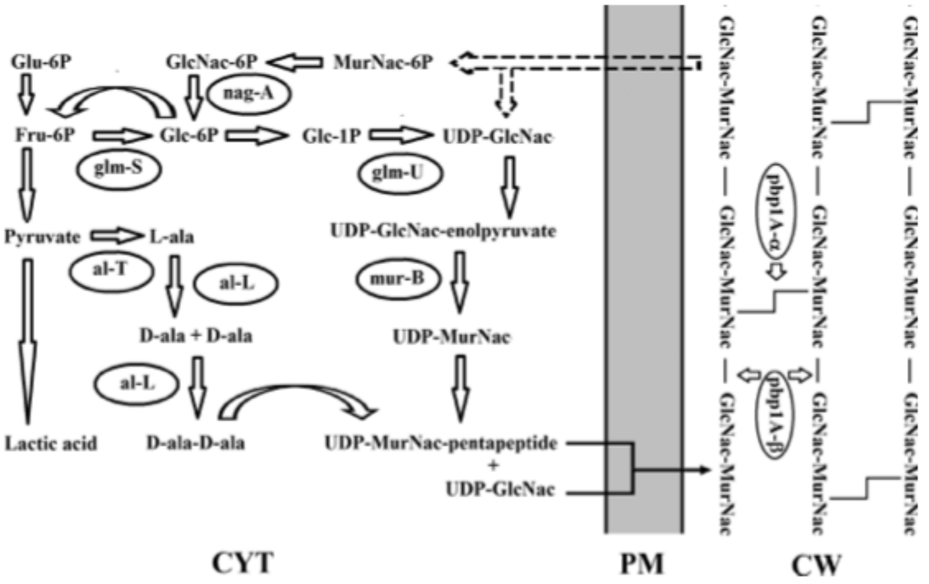

Fig. 4. Metabolic pathway of peptidoglycan biosynthesis. The enzymes whose genes were analysed in the present work are represented by an ellipse close to their catalyzed reactions. In the cytosol (CYT), carbon from the glucose enters the glycolytic pathway towards pyruvate to produce energy and then from pyruvate to lactic acid for NADH reoxidation. Alternatively can be diverted to cell wall precursor biosynthesis. The precursors of peptidoglycan are assembled in the cell wall monomer during the translocation process through the plasmatic membrane (PM) to the cell wall (CW). A glycosidic bond (—) is made to connect the new monomer in the growing peptidoglycan strand and a peptide bond (⌐) crosslinks close strands in a mature peptidoglycan. Glucose-6P (Glu-6P), fructose-6P (Fru-6P), pyruvate (Pyr), glucosamine (Glc), N-acetyl-glucosamine (GlcNac), N-acetyl-muramate (MurNac) and alanine (Ala) represent the ligants. Glucosamine synthase EC 2.6.1.16 (glm-S), bifunctional UDP-N-acetylglucosamine pyrophosphorylase EC 2.7.7.23/ glucosamine-1-phosphate N-acetyltransferase EC 2.3.1.157 (glm-U), UDP-N- acetylmuramate dehydrogenase EC 1.3.1.98 (mur-B), peptidoglycan transglycosylas PBP 1A EC 2.4.1.129 (pbp1A-β), DD-transpeptidase EC 3.4.16.4 (pbp1A-α), alanine aminotransferase EC 2.6.1.21 (al-T), alanine racemase EC 5.1.1.1 (al-R), alanine ligase EC 2.6.1.21 (al-L), and N-acetylglucosamine-6-phosphate deacetylase EC 3.5.1.25 (nag-A) are the enzymes involved.

of this gene was only observed upon exposure to HCl, while we observed down-regulation after exposure to organic acids (Fig. 5a). In addition, we evaluated the expression of the $\boldsymbol{\sigma}^{V}$-controlled *pbpX* gene that encodes for a penicillin-binding protein with D,D-carboxypeptidase activity that is involved in recycling of the alanine by cleaving the D-alanyl-D-alanine in stem peptides [15, 18] (Fig. 4). The results showed no significant change in its transcriptional level after treatment with any of the three acids (Fig. 5a). The other $\boldsymbol{\sigma}^{V}$ factor-regulated genes tested were those located in the operon *dlt* (*dltA-dltD*) that is responsible for the incorporation of D-alanine residues into the LTAs of the cell envelope [19]. This operon is responsible for resistance to cationic antimicrobials [20], such as vancomycin. When using the *dltD* gene as a representative of the *dlt* operon, the results showed down-regulation upon treatment with HCl and acetic acid and no change when the cells were exposed to lactic acid (Fig. 5a). Therefore, we concluded that in *L. vini* the transcription factor $\boldsymbol{\sigma}^{V}$ seems not to be the main regulator, or actually involved, in the genetic regulation of lysozyme resistance in response to acid stress (Fig. 1c).

The physiological role of the *dlt* operon is still poorly understood. Although the function of its corresponding enzymes and the chemical modification in the cell wall are quite well known, their biological consequences are not completely understood. The main information concerning the biological implications of this operon has been derived from mutant strains that are more susceptible to autolysis [18, 28], acid stress [40], antimicrobial agents such as vancomycin [49] and cationic antimicrobials [20]. In part, the mutant phenotype is due to the modification of the cell wall's overall charge, which becomes more negative than that of the wild-type, which makes the wall more susceptible to a positively charged compound such as a cationic antimicrobial or protons in the medium generated by the presence of acid compounds.

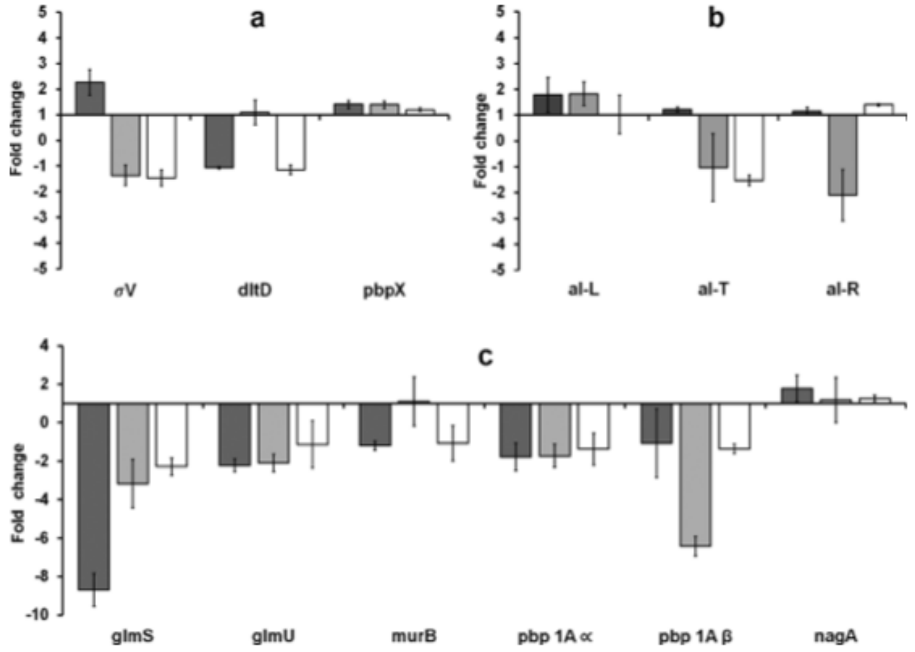

Fig. 5. Transcriptional profile of cell wall-related genes of *Lactobacillus vini* in response to treatment with three acidifying agents: hydrochloric acid at pH 3.5 (white columns), lactic acid (light grey columns) or acetic acid (dark grey columns). (a) Genes of the σ^V regulon. (b) Genes for D-alanine synthesis. (c) Genes for peptidoglycan biosynthesis. The results represent the average of three independent experiments with technical triplicates (±standard deviation). The significance of the data was assessed by an ANOVA test.

Genetic regulation of peptidoglycan metabolism

After assessing the transcriptional levels of genes involved in D-alanine incorporation in LTA, we evaluated the expression of genes involved in D-alanine biosynthesis: *al-L*, *al-R* and *al-T* (Fig. 4). *al-T* is involved in the endogenous production of L-alanine from pyruvate, in a transamination reaction using glutamate as an amino donor. *al-R* encodes a racemase that can conversely interconvert L-alanine to D-alanine. The *al-L* gene encodes a D-alanyl-D-alanine synthetase that is used in stem peptide synthesis. Analyses of the transcriptional level showed that the *al-L* gene was only marginally up-regulated, while the other two genes remained unchanged or showed down-regulation in response to the stress condition (Fig. 5b). Altogether, these results indicated that *L. vini* cells respond to the acidic environment by decreasing the transcription level of genes involved in D-alanine biosynthesis (*al* genes) and its incorporation into the cell envelope (*dlt* genes), which may result in a modification of the cell envelope that leads to increased resistance to lysozyme.

To complement this genetic analysis, we examined the transcription levels of genes involved in peptidoglycan precursor biosynthesis (*glmS*, *glmU* and *murB*) and extracellular peptidoglycan biosynthesis (*pbp1A*-like ORFs), as well as the gene involved in peptidoglycan reabsorption (*nagA*), in response to acidic stress (Fig. 4). Overall, all of these genes were down-regulated in acid-treated cells with respect to untreated cells (Fig. 5c), indicating a reduction in peptidoglycan biosynthesis as part of the cellular response to acidic stress. In Gram-positive bacteria the main regulation point of the peptidoglycan precursor biosynthesis pathway is the expression of the *glmS* gene [50]. This gene codes for glutamine-fructose-6-phosphate aminotransferase, the first enzyme in the pathway, and its regulation has a direct impact on the overall production of peptidoglycan precursor (Fig. 5). In lactobacilli,the down-regulation of *glmS* in response to cytoplasm acidifying agents, such as bile salt in *L. casei*, is observed [51]. Under this kind of stress, there is an increasing demand for ATP from the F_0F_1 extrusion pumps to maintain the internal pH. Hence, the down-regulation of *glmS* gene decreases the level of glutamine-fructose-6-phosphate aminotransferase activity and reduces the flux of carbon to the peptidoglycan biosynthetic pathway. As a consequence, all fructose-6P is driven towards glycolysis for higher production of ATP [51]. Our results showed that the expression of *glmS* was reduced by nine-, four-

and twofold in *L. vini* cells after exposure to HCl, lactic acid and acetic acid, respectively (Fig. 5c). This indicates that the biosynthesis of peptidoglycan is reduced in response to acidic stress. The preservation of the glutamine pool, which has a protective effect against acidic stress, as shown, should be a side-effect of this mechanism (Fig. 2).

The protein encoded by the *glmU* gene is a bifunctional enzyme that is responsible for the biosynthesis of UDP-GlcNAc, one of the precursors of the peptidoglycan structure (Fig. 4). Two sequential reactions are catalyzed: (1) its C-terminal domain transfers the acetyl group from acetyl coenzyme A to glucosamine-1-phosphate to produce GlcNAc-1-P; (2) afterwards, its N-terminal domain catalyzes a reaction that substitutes the phosphate group by uridine 5-monophosphate to ultimately produce UDP-GlcNAc. Our results showed that the *glmU* gene was down-regulated (twofold) after the treatment of cells with any of the three acids tested (Fig. 5c). Moreover, we measured the expression of the *murB* gene, whose proteins display UDP-N-acetylenolpyruvoylglucosamine reductase activity that produces UDP-N-acetylmuramic acid, as an important constituent of the bacterial cell wall [52]. This gene was also down-regulated (twofold) in HCl and remained unchanged upon weak organic acid treatment (Fig. 5c). Altogether, we can conclude that exposing *L. vini* cells to acidic stress by inorganic acid impacts on the regulation of key genes involved with the bacterial cell wall biosynthesis, which might lead to a reduction in the biosynthesis of this major cell component.

We also analysed the orthologous of genes coding for PBP 1A protein. This is a bifunctional protein comprising one catalytic domain that is involved in the transglycosylation reaction and one domain that is necessary for the transpeptidation reaction; both domains are required for the biosynthesis of extracellular peptidoglycan [17]. A search in the genome of both strains of *L. vini* revealed the presence of two ORFs (*pbp 1A-α* and *pbp 1A-β*) annotated as PBP 1A protein. These two theoretical proteins were half the size of PBP 1A from the model organism *Escherichia coli* (data not shown). Each one of these *L. vini* ORFs seemed to be responsible for encoding a peptide with one of the active domains of *E. coli* PBP 1A: *pbp1A-α* aligned to the C-terminal domain responsible for the transpeptidase activity of PBP 1A, while *pbp 1A-β* aligned to the N-terminal domain responsible for the transglycosylase activity of PBP 1A. This indicated that each protein is monofunctional in *L. vini* and might act co-ordinately for the biosynthesis of peptidoglycan in *L. vini* in the way that single bifunctional proteins do in other bacteria. This result was quite interesting and different from what has been reported for model bacterial species. Hence, we decided to analyse the structure of this gene in the closest phylogenetic LAB species of the salivarius group. For that purpose, we uploaded the genomes of *L. salivarius* strain UCC118 and *L. nagelii* strain DSM13675 to the RAST annotation tool and found that the PBP 1A gene codifies for a single protein with both domains in these LAB, as in *E. coli*. In addition, the PBP 1A gene was located downstream from the *recU* gene in all three LAB, but only in *L. vini* and *L. nagelii* was it followed by an endonuclease III gene. Therefore, PBP 1A seemed to have experienced a singular evolution in *L. vini* species that was not shared with the type species of the salivarius group and close species. Our results showed that both genes were down-regulated after cell exposure to any of the acids tested (Fig. 5c). Based on our previous results, we hypothesized that a reduction in the biosynthesis of peptidoglycan precursors, as indicated by down-regulation of the key genes reported above, might reduce the availability of substrate for PBP reactions. Consequently, the transcriptional activity of both *pbp 1A-α* and *pbp 1A-β* genes is reduced in response to acidic stress.

The genetic analyses presented above indicated a reduced synthesis of peptidoglycan, possibly due to the high demand for maintenance ATP, which is required for proton extrusion. In order to meet this demand upon more intense cytoplasm acidification, the carbon trapped in the cell wall can be recycled and used for energy production in the glycolytic pathway. The *nagA* is one of the genes involved in the cell wall reabsorption pathway through the production of N-acetylglucosamine-6-phosphate deacetylase (Fig. 4). This enzyme breaks down GlcNAc-6-P to produce glucosamine 6-phosphate and acetate [22], somehow reverting the reaction catalyzed by the *glmU* gene product referred to above and providing intermediate molecules that can provide carbon for glycolysis, such as fructose-6-phosphate. In the present work we report a twofold up-regulation of this cell wall catabolic gene *nagA* upon treatment with HCl (Fig. 5c), the condition that promoted the highest negative effect on the expression of the cell wall biosynthetic genes *glmS* and *murB* (Fig. 5c). In *L. casei* [51] and *L. rhamnosus* [53], the protein NAGA was reported to be accumulated in the cytoplasm in stress conditions that resulted in cytoplasm acidification. On the other hand, in *L. casei* we also observed decreasing levels of GLM-S protein [51]. Therefore, stress induced by HCl might be triggering a response mechanism that reduces the biosynthetic activity of the bacterial cell wall in order to save carbon to address the increasing energy demand. Moreover, it might accelerate cell wall reabsorption to supply the glycolytic flux with hexose to increase energy production.

Taken together, it can be concluded that in *L. vini* the acid stress response is a complex biological phenomenon that acts to co-ordinate two complementary, albeit independent, mechanisms. Since the control of internal pH seems to be the major problem bacterial cells must deal with, all efforts must be made to neutralize the excess of protons in the cytoplasm caused by the external presence of acids. Alkalinization reactions for the maintenance of internal pH, together with the energy-demanding reaction for proton extrusion, might contribute to the anticipated entry into the stationary cell growth phase. This reduces the deposition of the components of the cell wall while signalling for their reabsorption. As consequence, the cell wall structure is remodelled to become resistant to the hydrolytic activity of lysozyme. Altogether, these physiological changes might improve cell survival and competitiveness in stressful environments such as the industrial one. This is a phenomenon that has rarely been described among lactobacilli and one that has never been associated with acid stress. This makes this feature, to date, the signature of *L. vini* acid stress response.

Funding information

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

This article does not contain any studies with human participants or animals performed by the authors.

References

1. **Lucena BT, dos Santos BM, Moreira JL, Moreira AP, Nunes AC *et al.*** Diversity of lactic acid bacteria of the bioethanol process. *BMC Microbiol* 2010;10:298.
2. **Beckner M, Ivey ML, Phister TG.** Microbial contamination of fuel ethanol fermentations. *Lett Appl Microbiol* 2011;53:387–394.
3. **Basso TO, Gomes FS, Lopes ML, de Amorim HV, Eggleston G *et al.*** Homo- and heterofermentative *lactobacilli* differently affect sugarcane-based fuel ethanol fermentation. *Antonie van Leeuwenhoek* 2014;105:169–177.
4. **Rich JO, Leathers TD, Bischoff KM, Anderson AM, Nunnally MS.** Biofilm formation and ethanol inhibition by bacterial contaminants of biofuel fermentation. *Bioresour Technol* 2015;196:347–354.
5. **Muthaiyan A, Limayem A, Ricke SC.** Antimicrobial strategies for limiting bacterial contaminants in fuel bioethanol fermentations. *Prog Energy Combust Sci* 2011;37:351–370.
6. **Mendonça AA, de Lucena BT, de Morais MM, de Morais MA.** First identification of Tn916-like element in industrial strains of *Lactobacillus vini* that spread the *tet-M* resistance gene. *FEMS Microbiol Lett* 2016;363:fnv240.
7. **Murphree CA, Heist EP, Moe LA.** Antibiotic resistance among cultured bacterial isolates from bioethanol fermentation facilities across the United States. *Curr Microbiol* 2014;69:277–285.
8. **Rasmussen ML, Koziel JA, Jane JL, Pometto AL.** Reducing bacterial contamination in fuel ethanol fermentations by ozone treatment of uncooked corn mash. *J Agric Food Chem* 2015;63:5239–5248.
9. **Costa MAS, Cerri BC, Ceccato-Antonini SR.** Ethanol addition enhances acid treatment to eliminate *Lactobacillus fermentum* from the fermentation process for fuel ethanol production. *Lett Appl Microbiol* 2018;66:77–85.
10. **Tiukova I, Eberhard T, Passoth V.** Interaction of *Lactobacillus vini* with the ethanol-producing yeasts *Dekkera bruxellensis* and *Saccharomyces cerevisiae*. *Biotechnol Appl Biochem* 2014;61:40–44.
11. **Skinner KA, Leathers TD.** Bacterial contaminants of fuel ethanol production. *J Ind Microbiol Biotechnol* 2004;31:401–408.
12. **Rodas AM, Ferrer S, Pardo I.** Polyphasic study of wine *Lactobacillus* strains: taxonomic implications. *Int J Syst Evol Microbiol* 2005;55:197–207.
13. **Barre P.** Identification of thermobacteria and homofermentative, thermophilic, pentose-utilizing *lactobacilli* from high temperature fermenting grape musts. *J Appl Bacteriol* 1978;44:125–129.
14. **Luckwu de Lucena BT, Silva GG, Manoel dos Santos B, Dias GM, Amaral GR *et al.*** Genome sequences of the ethanol-tolerant *Lactobacillus vini* strains LMG 23202T and JP7.8.9. *J Bacteriol* 2012;194:3018.
15. **Scheffers DJ, Pinho MG.** Bacterial cell wall synthesis: new insights from localization studies. *Microbiol Mol Biol Rev* 2005;69:585–607.
16. **Münch D, Sahl HG.** Structural variations of the cell wall precursor lipid II in Gram-positive bacteria - Impact on binding and efficacy of antimicrobial peptides. *Biochim Biophys Acta* 2015;1848:3062–3071.
17. **Sauvage E, Terrak M.** Glycosyltransferases and Transpeptidases/Penicillin-Binding Proteins: Valuable Targets for New Antibacterials. *Antibiotics* 2016;5:12–27.
18. **Chapot-Chartier MP, Kulakauskas S.** Cell wall structure and function in lactic acid bacteria. *Microb Cell Fact* 2014;13 Suppl 1:S9.
19. **Weidenmaier C, Peschel A.** Teichoic acids and related cell-wall glycopolymers in Gram-positive physiology and host interactions. *Nat Rev Microbiol* 2008;6:276–287.
20. **Abi Khattar Z, Rejasse A, Destoumieux-Garzón D, Escoubas JM, Sanchis V *et al.*** The dlt operon of *Bacillus cereus* is required for resistance to cationic antimicrobial peptides and for virulence in insects. *J Bacteriol* 2009;191:7063–7073.
21. **Bernard E, Rolain T, Courtin P, Guillot A, Langella P *et al.*** Characterization of O-acetylation of N-acetylglucosamine: a novel structural variation of bacterial peptidoglycan. *J Biol Chem* 2011;286:23950–23958.
22. **Reith J, Mayer C.** Peptidoglycan turnover and recycling in Gram-positive bacteria. *Appl Microbiol Biotechnol* 2011;92:1–11.
23. **Vollmer W, Joris B, Charlier P, Foster S.** Bacterial peptidoglycan (murein) hydrolases. *FEMS Microbiol Rev* 2008;32:259–286.
24. **Davis KM, Weiser JN.** Modifications to the peptidoglycan backbone help bacteria to establish infection. *Infect Immun* 2011;79:562–570.
25. **Bera A, Biswas R, Herbert S, Kulauzovic E, Weidenmaier C *et al.*** Influence of wall teichoic acid on lysozyme resistance in *Staphylococcus aureus*. *J Bacteriol* 2007;189:280–283.
26. **Guariglia-Oropeza V, Helmann JD.** *Bacillus subtilis* σ^{V} confers lysozyme resistance by activation of two cell wall modification pathways, peptidoglycan O-acetylation and D-alanylation of teichoic acids. *J Bacteriol* 2011;193:6223–6232.
27. **Hastie JL, Williams KB, Bohr LL, Houtman JC, Gakhar L *et al.*** The anti-sigma factor RsiV Is a bacterial receptor for lysozyme: co-crystal structure determination and demonstration that binding of lysozyme to RsiV is required for σ^{V} activation. *PLoS Genet* 2016;12:e1006287–30.
28. **Palumbo E, Deghorain M, Cocconcelli PS, Kleerebezem M, Geyer A *et al.*** D-alanyl ester depletion of teichoic acids in *Lactobacillus plantarum* results in a major modification of lipoteichoic acid composition and cell wall perforations at the septum mediated by the *Acm2* autolysin. *J Bacteriol* 2006;188:3709–3715.
29. **Piuri M, Sanchez-Rivas C, Ruzal SM.** Cell wall modifications during osmotic stress in *Lactobacillus casei*. *J Appl Microbiol* 2005;98:84–95.
30. **Narendranath NV, Hynes SH, Thomas KC, Ingledew WM.** Effects of *lactobacilli* on yeast-catalyzed ethanol fermentations. *Appl Environ Microbiol* 1997;63:4158–4163.
31. **Basso LC, Basso TO, Rocha SN.** Ethanol production in Brazil: the industrial process and its impact on yeast fermentation. In: *Biofuel Production-Recent Developments and Prospects*. pp. 85–100.
32. **Wu C, Zhang J, Du G, Chen J.** Aspartate protects *Lactobacillus casei* against acid stress. *Appl Microbiol Biotechnol* 2013;97:4083–4093.
33. **Aziz RK, Bartels D, Best AA, Dejongh M, Disz T *et al.*** The RAST Server: rapid annotations using subsystems technology. *BMC Genomics* 2008;9:75.

34. **Marco ML, Kleerebezem M.** Assessment of real-time RT-PCR for quantification of *Lactobacillus plantarum* gene expression during stationary phase and nutrient starvation. *J Appl Microbiol* 2008; 104:587–594.
35. **Ginzinger DG.** Gene quantification using real-time quantitative PCR. *Exp Hematol* 2002;30:503–512.
36. **Stenico V, Baffoni L, Gaggìa F, Biavati B.** Validation of candidate reference genes in *Bifidobacterium adolescentis* for gene expression normalization. *Anaerobe* 2014;27:34–39.
37. **Løvdal T, Saha A.** Reference gene selection in *Carnobacterium maltaromaticum, Lactobacillus curvatus,* and *Listeria innocua* subjected to temperature and salt stress. *Mol Biotechnol* 2014;56: 210–222.
38. **Huggett J, Dheda K, Bustin S, Zumla A.** Real-time RT-PCR normalisation; strategies and considerations. *Genes Immun* 2005;6: 279–284.
39. **Passoth V, Blomqvist J, Schnürer J.** *Dekkera bruxellensis* and *Lactobacillus vini* form a stable ethanol-producing consortium in a commercial alcohol production process. *Appl Environ Microbiol* 2007;73:4354–4356.
40. **Cotter PD, Hill C.** Surviving the acid test: responses of gram-positive bacteria to low pH. *Microbiol Mol Biol Rev* 2003;67:429–453.
41. **de Lucena RM, Elsztein C, Simões DA, de Morais MA.** Participation of CWI, HOG and Calcineurin pathways in the tolerance of *Saccharomyces cerevisiae* to low pH by inorganic acid. *J Appl Microbiol* 2012;113:629–640.
42. **Logardt IM, Neujahr HY.** Lysis of modified walls from *Lactobacillus* fermentum. *J Bacteriol* 1975;124:73–77.
43. **Goffin P, van de Bunt B, Giovane M, Leveau JH, Höppener-Ogawa S *et al.*** Understanding the physiology of *Lactobacillus plantarum* at zero growth. *Mol Syst Biol* 2010;6:413.
44. **Papadimitriou K, Á A, Bron PA, De AM, Gobbetti M *et al.*** *Stress Physiology of Lactic Acid Bacteria*, vol. 80; 2016. pp. 837–890.
45. **Siegumfeldt H, Björn Rechinger K, Jakobsen M.** Dynamic changes of intracellular pH in individual lactic acid bacterium cells in response to a rapid drop in extracellular pH. *Appl Environ Microbiol* 2000;66:2330–2335.
46. **Arena ME, Manca de Nadra MC, Muñoz R.** The arginine deiminase pathway in the wine lactic acid bacterium *Lactobacillus hilgardii* X1B: structural and functional study of the arcABC genes. *Gene* 2002;301:61–66.
47. **Teixeira JS, Seeras A, Sanchez-Maldonado AF, Zhang C, Su MS *et al.*** Glutamine, glutamate, and arginine-based acid resistance in *Lactobacillus reuteri. Food Microbiol* 2014;42:172–180.
48. **Molenaar D, Bosscher JS, Ten Brink B, Driessen AJ, Konings WN.** Generation of a proton motive force by histidine decarboxylation and electrogenic histidine/histamine antiport in *Lactobacillus buchneri. J Bacteriol* 1993;175:2864–2870.
49. **Peschel A, Vuong C, Otto M, Götz F.** The D-alanine residues of *Staphylococcus aureus* teichoic acids alter the susceptibility to vancomycin and the activity of autolytic enzymes. *Antimicrob Agents Chemother* 2000;44:2845–2847.
50. **Komatsuzawa H, Fujiwara T, Nishi H, Yamada S, Ohara M *et al.*** The gate controlling cell wall synthesis in *Staphylococcus aureus. Mol Microbiol* 2004;53:1221–1231.
51. **Alcántara C, Zúñiga M.** Proteomic and transcriptomic analysis of the response to bile stress of *Lactobacillus casei* BL23. *Microbiology* 2012;158:1206–1218.
52. **Barreteau H, Kovac A, Boniface A, Sova M, Gobec S *et al.*** Cytoplasmic steps of peptidoglycan biosynthesis. *FEMS Microbiol Rev* 2008;32:168–207.
53. **Koskenniemi K, Laakso K, Koponen J, Kankainen M, Greco D *et al.*** Proteomics and transcriptomics characterization of bile stress response in probiotic *Lactobacillus rhamnosus* GG. *Mol Cell Proteomics* 2011;10:M110.002741.

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