CÍNTIA LACERDA RAMOS

CARACTERIZAÇÃO DE *Lactobacillus* spp. E DESENVOLVIMENTO DE UM SISTEMA DE SIMULAÇÃO DE SOBREVIVÊNCIA BACTERIANA NO TRATO GASTROINTESTINAL

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós Graduação em Microbiologia Agrícola, área de concentração em Processos Fermentativos Aplicados e Agroindústria, para a obtenção do título de Doutor.

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RESUMO

O presente trabalho foi realizado com o objetivo de estudar as características probióticas de bactérias ácido-láticas (BAL) isoladas de três produtos/processos alimentícios (linguiça fresca, fermentação de cacau e cauim). Um modelo do trato gastrointestinal (TGI) para avaliação das condições fisiológicas celulares por meio de análises de pH intracelular (pH_i) foi desenvolvido. O modelo foi empregado para avaliação de dois potenciais probióticos selecionados, *Lactobacillus plantarum* UFLA CH3 e *Lb. brevis* UFLA FFC199. Inicialmente, 234 BAL foram submetidas a teste de sobrevivência em pH 2,0, sendo, então, selecionadas 51. Estas foram identificadas como *Lb. fermentum* (34 isolados), *Lb. plantarum* (10) e *Lb. brevis* (7). Com base nos resultados de tolerância à bile (0,3 %), habilidade para autoagregação e propriedades hidrofóbicas, os isolados *Lb. fermentum* (UFLA CH58), *Lb. plantarum* (UFLA CH3, UFLA CH41 e UFLA SAU96) e *Lb. brevis* (UFLA SAU105 e UFLA FFC199) foram selecionados. *Lb. plantarum* UFLA CH41 apresentou a maior capacidade de coagregação com *Escherichia coli*, enquanto que *Lb. brevis* (UFLA SAU105 e UFLA FFC199) e *Lb. fermentum* UFLA CH58 exibiram atividade antagônica contra os patógenos *Listeria monocytogenes* e *Staphylococcus aureus*. Em relação à adesão às células Caco-2, *Lb. plantarum* UFLA CH3 e UFLA CH41 e *Lb. brevis* UFLA FFC199 (1,6%, 1,1% e 0,9%, respectivamente) mostraram resultados similares ao do probiótico comercial *Lb. rhamnosus* GG (1,5%). Estes isolados também foram capazes de aumentar significativamente a resistência elétrica da barreira transepitelial (TEER) das células Caco-2 em 24 horas (p<0,05). Contudo, por apresentarem características desejáveis para uso probiótico, *Lb. plantarum* CH3 (cacau) e *Lb. brevis* FFC199 (cauim) foram selecionados e estudados no modelo do TGI desenvolvido. Análises do pH_i foram determinadas empregando-se a metodologia *fluorescence ratio imaging microscopy* (FRIM). Foi observada heterogeneidade nas populações, com valores de pH_i variando de 6,5–7,5 e 6,5-8,0 ou maior, durante passagem da saliva (pH 6,4) e do suco intestinal (pH 6,4), respectivamente. Exposição ao suco gástrico (pH 3,5) gerou duas subpopulações, uma com pH_i variando de 3,5-4,5 e outra, de 4,5-5,6. O isolado *Lb. brevis* UFLAFFC199 apresentou valores de pH_i mais próximos ao do pH_{ex}, quando uma solução nutritiva foi adicionada ao suco gástrico, o que não foi observado para *Lb. plantarum* UFLACH3. O modelo do TGI desenvolvido, empregando a técnica de FRIM, capacitou o monitoramento em tempo real da homeostase do pH de células únicas. Os isolados *Lb. plantarum* UFLA CH3 e *Lb. brevis* UFLA FFC199 apresentaram potenciais características para uso probiótico, sendo capazes de sobreviver às condições do TGI, mantendo-se viáveis e vitais.

Palavras-chave: Probiótico. *Lactobacillus* spp. Adesão. TEER. pH_i. FRIM. Modelo gastro-intestinal *in vitro*.

ABSTRACT

This work aimed to study the probiotic characteristics of lactic acid bacteria (LAB) isolated from three food products / processes (fresh sausage, cocoa fermentation and cauim). A model of the gastrointestinal (GI tract to evaluate the physiological conditions of cells by intracellular pH (pH_i) analysis was developed. The model was used to evaluate two selected potential probiotic *Lactobacillus plantarum* UFLA CH3 and *Lb. brevis* UFLA FFC199. Initially, 234 LAB were subject to pH 2.0 test, and then 51 were selected. They were identified as *Lb. fermentum* (34 isolates), *Lb. plantarum* (10) and *Lb. brevis* (7). Based on the results of bile tolerance (0.3%), ability to auto-aggregation and hydrophobic properties, the isolate *Lb. fermentum* (UFLA CH58), *Lb. plantarum* (UFLA CH3, UFLA CH41 and UFLA SAU96) and *Lb. brevis* (UFLA SAU105 and UFLA FFC199) were selected. *Lb. plantarum* UFLA CH41 showed the highest ability to co-aggregate with *Escherichia coli*, while *Lb.brevis* (UFLA SAU105 and UFLA FFC199) and *Lb. fermentum* UFLA CH58 exhibited antagonistic activity against the pathogens *Listeria monocytogenes* and *Staphylococcus aureus*. Regarding adherence to Caco-2 cells, *Lb. plantarum* UFLA CH3 and UFLA CH41 and *Lb. brevis* UFLA FFC199 showed similar results (1.6, 1.1 and 0.9%, respectively) to the commercial probiotic *Lb. rhamnosus* GG (1.5%). These isolates were also able to significantly increase the transepithelial electrical resistance (TEER) of Caco-2 cells over 24 h ($p < 0.05$). Therefore, due to their potential probiotic characteristics, *Lb. plantarum* UFLA CH3 (cocoa) and *Lb. brevis* UFLA FFC199 (cauim) were selected and applied in the GI tract model developed in the present study. Analyses of pH_i were determined employing Fluorescence Ratio Imaging Microscopy (FRIM). We observed heterogenous populations, with pH_i values ranging from 6.5-7.5 and 6.5-8.0 or higher during passage of saliva (pH 6.4) and intestinal juice (pH 6.4), respectively. Exposure to gastric juice (pH 3.5) showed two sub-populations with pH_i ranging from 3.5-4.5 and 4.5-5.6. The isolate *Lb. brevis* UFLA FFC199 showed pH_i values closest to pH_{ex} when a nutrient solution was added to gastric juice, which was not observed for *Lb. plantarum* UFLA CH3. The GI model developed employing FRIM, enabled a real-time monitoring of pH homeostasis of single cells. The isolates *Lb. plantarum* UFLA CH3 and *Lb. brevis* UFLA FFC199 showed characteristics for potential probiotic use, being able to survive the conditions of the GI tract maintaining viable and vital.

Keywords: Probiotic. *Lactobacillus* spp. Adhesion.TEER.pHi. FRIM.Gastro-intestinal model *in vitro*.

SUMÁRIO

PRIMEIRA PARTE

1 INTRODUÇÃO

A população, de modo geral, está mais consciente da relação existente entre alimentação e saúde. Sendo assim, a indústria tem buscado novas alternativas para o desenvolvimento de alimentos e de suplementos alimentares de boa aceitabilidade e com ingredientes capazes de promover a saúde.

Probióticos são microrganismos vivos que, após ingestão, se tornam parte da microbiota do cólon, pelo menos temporariamente, e são utilizados com o objetivo de melhorar a saúde e o bem-estar do hospedeiro. Grande número de usos de probióticos para o combate de doenças gastrintestinais vem sendo proposto, incluindo modulação da imunidade da mucosa intestinal, além da prevenção e do tratamento de infecções intestinais.

As bactérias ácido-láticas (BAL) vêm sendo utilizadas como probióticos em diversos produtos ou como suplemento alimentar, devido ao comprovado benefício provocado no organismo hospedeiro. Capacidade de regular a microbiota intestinal, inibição da adesão de microrganismos patogênicos no trato gastrintestinal (TGI), redução do colesterol sérico, aumento da resposta imune do hospedeiro e, ainda, redução do risco de doenças alérgicas, da intolerância à lactose e da atividade carcinogênica são exemplos comprovados da atividade funcional dos probióticos. As bactérias probióticas geralmente estudadas pertencem aos gêneros *Lactobacillus* e *Bifidobacterium*, que apresentam uma longa história de uso seguro em alimentos.

No processo de seleção de um microrganismo probiótico, devem-se considerar a origem da estirpe, a capacidade de adesão à mucosa intestinal, a tolerância a ácidos, a tolerância à bile, o antagonismo contra patógenos e o

histórico de não patogenicidade (FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS - FAO, 2002).

As bactérias probióticas são incorporadas, principalmente, a alimentos fermentados, sendo os produtos lácteos seus principais carreadores. Uma vez que as estirpes de *Lactobacillus* spp. isoladas de diferentes produtos não são as mesmas além do fato de que cada bactéria desempenha diferente função no corpo, torna-se importante o estudo de diferentes estirpes, isoladas das mais diversas fontes.

Devido ao importante uso de BAL como probióticos, estudos dos mecanismos utilizados por essas bactérias para sobreviverem ao ambiente hostil imposto pelo TGI tornam-se imprescindíveis. Estresse ácido e a presença de bile no TGI dificultam a sobrevivência dos mais diversos grupos de microrganismos, incluindo as BAL.

A homestase de pH intracelular (pH_i) está intimamente ligada às condições fisiológicas celulares, já que a maioria das enzimas é dependente dos valores de pH para apresentar sua atividade ótima. A *fluorescence ratio imaging microscopy* (FRIM), ou técnica de microscopia de fluorescência, vem sendo utilizada com sucesso para análises de pH_i de células individuais microbianas. Diversos modelos simulando o TGI para estudos do comportamento microbiano vêm sendo desenvolvidos, no entanto, nenhum deles possibilita a obtenção de resultados para células individualmente e, sim, populacional. Modelos empregando o método de FRIM têm a vantagem de apresentarem resultados para células individualmente, possibilitando a observação das diferentes respostas entre as células e, então, a formação de subpopulações.

Inicialmente, este trabalho foi realizado com os objetivo de identificar e avaliar as propriedades probióticas de estirpes de *Lactobacillus* spp. isoladas de linguiça fresca industrial, fermentação de cacau e cauim (bebida fermentada indígena); desenvolver um novo sistema para o monitoramento, em tempo real,

das condições fisiológicas celulares nas condições do TGI por meio de avaliações do pH_i pela técnica de FRIM e, por fim, avaliar as condições fisiológicas de potenciais probióticos selecionados no novo modelo do TGI desenvolvido neste trabalho.

2 REFERENCIAL TEÓRICO

2.1 A microbiota do trato gastrintestinal

O corpo humano tem diversas comunidades microbianas que vivem em relações simbióticas. Estas relações podem ser mutualísticas (beneficiando microrganismos e hospodeiros), comensais (beneficiando somente microrganismos) ou patogênicas (beneficiando microrganismos causando prejuízo aos hospedeiros). O trato gastrintestinal (TGI) é um sistema complexo que se estima conter mais de 500 espécies e 100 trilhões de microrganismos, o que corresponde a, aproximadamente, dez vezes mais células que o número total de células do corpo (BACKHED et al., 2005; ECKBURG et al.,2005). A microbiota intestinal aulixia o organimo na proteção contra enteropatógenos, na absorção de nutrientes e de energia da dieta, além de contribuir para o funcionamento do sistema imune.

Dados sobre a microbiota do TGI surgiram a partir do uso de metolodologias baseadas no cultivo, o que restringe os resultados, selecionando-se apenas isolados capazes de crescer sob as condições laboratoriais. Nas últimas décadas, muito se tem avançado nesse sentido, devido ao uso de metodologias moleculares independentes de cultivo, permitindo a descoberta de novas espécies associadas a este ambiente. Este avanço possibilitou determinar que a maioria das bactérias presentes no TGI pertence aos filos Bacterioides e Firmicutes (BACKHED et al., 2005; ECKBURG et al., 2005). Os métodos independentes de cultivo têm demonstrado grande diversidade microbiana no TGI e estas comunidades microbianas apresentam grandes variações, de acordo com o tempo e as populações hospedeiras (CLAESSON et al., 2009). O potencial de oxirredução positivo no intestino dos neonatos favorece a presença de bactérias, como *Escherichia coli*, *Enterococcus* spp., *Staphylococcus* spp. e

Streptococcus spp. Posteriormente, com a diminuição do teor de oxigênio, o ambiente torna-se propício ao crescimento de bactérias anaeróbias obrigatórias, favorecendo a proliferação de *Bifidobacterium* spp., *Bacterioides* spp. e *Clostridium* spp. (MACKIE; SGHIR; GASKINS, 1999). Bactérias do gênero *Lactobacillus* e *E. coli* encontram-se em alta proporção na microbiota intestinal de crianças e adultos (MITSUOKA, 1996). Representantes dos filos Actinobacteria, Proteobacteria e Verrucoicrobia, embora já detectados no TGI humano, são, geralmente, encontrados em menor frequência neste ambiente (ECKBURG et al., 2005). Bactérias archea metanogênicas (principalmente *Methanobrevibacter smithii*), eucariotos (principalmente leveduras) e vírus (fagos) estão também presentes no TGI (REYES et al., 2010).

Variações das comunidades microbianas presentes no TGI, ao longo do tempo, são influenciadas pela dieta, a idade, o estado fisiológico do hospedeiro (ex.: estresse, gravidez) e o uso de antibióticos (KLIGLER; HANAWAY; COHRSSEN, 2007; SERINO et al., 2009). Um grupo de pesquisadores da Universidade de Washington nos Estados Unidos demonstrou que pessoas que perderam peso por meio de dietas pobres em gorduras e carboidratos reduziram os níveis de adiposidade em conjunto com a taxa de membros dos filos Firmicutes a Bacterioides (LEY et al., 2006). Estudos também revelam que a microbiota do intestino de mamíferos é mais similar dentro das espécies que em membros de diferentes espécies, mesmo quando há uma grande separação geográfica entre os membros de mamíferos da mesma espécie (LEY et al., 2008). Estes estudos demonstram uma clara coevolução do hospedeiro e sua microbiota e a importante contribuição da microbiota intestinal para a saúde humana (O'FLAHERTY; KLAENHAMMER, 2010).

2.2 BAL: taxonomia e funcionalidade

BAL representa um grupo de bactérias heterogêneas e que partilham muitas características fisiológicas, dentre elas destaca-se a capacidade de produzir ácido lático durante a fermentação. Taxonomicamente, espécies de BAL são encontradas em dois distintos filos: Firmicutes e Actinobacteria. Dentro do filo Firmicutes, as BAL pertencem à ordem *Lactobacillales* e incluem, entre outros, os seguintes gêneros: *Aerococcus*, *Alloiococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Symbiobacterium*, *Tetragenococcus*, *Vagococcus* e *Weissella*, que são todos organismos com baixo conteúdo GC (31-49%). Dentro do filo Actinobacteria, as BAL pertencem aos gêneros *Atopobium* e *Bifidobacterium*, com um teor de GC de 36%-46% e 58%-61%, respectivamente (KLAENHAMMER et al., 2005; PFEILER; KLAENHAMMER, 2007).

De modo mais geral, o termo BAL não reflete uma classe filogenética, mas sim as capacidades metabólicas deste grupo bacteriano heterogêneo, dos quais o mais importante é a capacidade de fermentar açúcares principalmente em ácido láctico. BAL são também caracterizadas por serem gram-positivas, catalase negativas (podem apresentar pseudocatalase), anaeróbias aerotolerantes, não esporulantes, geralmente motilidade negativa e ácido-tolerantes (PFEILER; KLAENHAMMER, 2007).

BAL são amplamente utilizadas em diversas aplicações industriais, como culturas iniciadoras em indústrias de alimentos fermentados, como probióticos em suplementos alimentares, além de agentes de bioconversão. Vários alimentos fermentados estão relacionados à atividade das BAL, proporcionando qualidade no *flavour*, na textura e na conservação destes alimentos. Os gêneros/espécies mais importantes para aplicações industriais são:

Lactobacillus (leite, carne, vegetal, cereal), *Lactococcus* (leite), *Leuconostoc* (leite, vegetal), *Pediococcus* (carne, vegetal), *Oenococcus oeni* (vinho) e *Streptococcus thermophilus* (leite) (KLAENHAMMER et al., 2005).

Plantas e animais, e o correspondente produto alimentício fermentado, são, geralmente, fontes nas quais se encontram as BAL (GIRAFFA, 2012). Algumas espécies também ocorrem nos tratos respiratório, intestinal e genital de humanos e animais. A capacidade para colonizar uma variedade de habitats é consequência direta da versatilidade metabólica deste grupo de bactérias. Contudo, as BAL são empregadas, ao longo de décadas, na conservação de alimentos, sendo amplamente consumidas por humanos e possuindo o status de *generally recognized as safe* (GRAS), isto é, seguras para o consumo (KLAENHAMMER et al., 2005).

2.3 BAL como probióticos

As BAL têm importante função na produção de alimentos, devido à sua contribuição positiva para o sabor e a conservação do produto final. Além das fermentações tradicionais, BAL são extensivamente utilizadas como agentes preservantes, acidulantes e flavorizantes em alimentos, como intermediário em fabricações de fármacos e cosméticos (ex. curativo cirúrgico), na fabricação de polímeros ácido polilático biodegradáveis (LIU, 2003) e para o desenvolvimento de probióticos.

O termo probiótico significa "por vida" e a Organização Mundial da Saúde (OMS) define probióticos como "microrganismos vivos que, quando administrados em quantidades adequadas, conferem benefícios à saúde do hospedeiro". Pesquisas na área de probióticos têm sido amplamente realizadas e progressos importantes relacionados à seleção e à caracterização de culturas probióticas vêm sendo obtidos, confirmando os benefícios à saúde associados a

eles. Tradicionalmente, alimentos fermentados são as principais fontes de probióticos e, portanto, um dos principais suplementos da dieta do mundo moderno.

BAL é o mais importante grupo de microrganismos comercialmente utilizados como culturas iniciadoras para a fabricação de produtos lácteos probióticos (HEENAN et al., 2002). Representantes dos gêneros *Lactobacillus* e *Bifidobacterium* são os mais amplamente utilizados e estudados como bactérias probióticas. Estas cumprem os critérios exigidos para serem selecionadas como probióticos, como, por exemplo, apresentar resistência às enzimas da cavidade oral; sobrevivência ao longo do TGI, chegando ao local de atuação num estado fisiológico viável e a aderência à superfície da célula hospedeira (OTERO; OCANA; MACIAS, 2004). Atualmente, os probióticos são consumidos em produtos lácteos fermentados ou como culturas liofilizadas. Os probióticos, ao atingirem a parte inferior do intestino delgado e cólon, colonizam e multiplicam-se para exercer seus efeitos benéficos. Os benefícios à saúde proporcionados pelas BAL variam desde a regulação da microbiota intestinal até a modulação da resposta imune. Melhora do quadro de intolerância à lactose, redução no risco de diarreia provocada por bactérias e vírus patogênicos, bem como redução do colesterol sérico são alguns outros efeitos positivos obtidos com o consumo de probiótico (ROBERFROID, 2000).

Na Tabela 1 mostram-se alguns dos produtos probióticos disponíveis no mercado, a BAL probiótica usada em cada preparação e os benefícios proporcionados à saúde.

Os mecanismos pelos quais as BAL diferencialmente modulam as funções da célula hospedeira dependem dos constituintes da parede celular das bactérias e seus correspondentes receptores nas células do hospedeiro. A capacidade das bactérias de colonizarem as superfícies da mucosa pela aderência às células epiteliais do TGI e as proteínas da matriz extracelular depende da

hidrofobicidade da superfície celular. A tolerância a ácidos e à bile auxilia na sobrevivência sob as severas condições físico-químicas impostas pelo ambiente do TGI (ZAREBA et al., 1997). As interações das células probióticas com a camada de mucina das células epiteliais do hospedeiro podem aumentar o tempo de retenção do probiótico no hospedeiro (OTERO; OCANA; MACIAS, 2004). A atividade antimicrobiana das BAL é atribuída à produção de ácidos orgânicos (CABO; BRABER; KOENRAAD, 2002; LIND et al., 2007) e/ou bacteriocinas (MAGNUSSON; SCHNURER, 2001; STROM et al., 2002; TALARICO et al., 1988). Bacteriocinas de menos de 20 kDa, geralmente, causam a despolarização da membrana das células alvo e/ou inibem a síntese da parede celular, e aquelas com mais de 20 kDa, geralmente, degradam a camada de mureína (SETTANNI; CORSETTI, 2008). A reuterina, um agente de amplo espectro antimicrobiano produzido por *L. reuteri*, afeta a síntese proteica, impedindo o crescimento do patógeno (TALARICO; DOBROGOSZ, 1989; VOLLENWEIDER; LACROIX, 2004). Os ácidos orgânicos, na sua forma hidrofóbica não dissociada, difundem-se através da membrana celular dos microrganismos patogênicos, neutralizando o potencial electroquímico e aumentando sua permeabilidade, resultando, então, na bacteriostase e na morte do patógeno (DALIE; DESCHAMPS; RICHARD-FORGET, 2010; DIVYA; VARSHA; NAMPOOTHIRI, 2012).

Tabela 1 Exemplos de probióticos disponíveis no mercado. Modificada de Divya et al. (2012)

Nome comercial	**Organismo**	**Benefícios à saúde**	**Fonte**
Culturelle probiotic (Amerifit Brands)	*Lactobacillus* GG	Restaura equilíbrio da microbiota intestinal e estimula sistema imune	www.culturelle.com
Kyo-Dophilus (Kyolic)	*L. acidophilus, B. bifidum, B. longum*	Regulação da função intestinal	www.kyolic.com
Dannon DanActive	*L. casei* Immunitas	Recompõe microbiota após diarreia associada à antibióticose estimula sistema imune	www.dannon.com
RepHresh Pro-B	*L. rhamnosus, L. reuteri*	Mantem microbiotavaginal	www.pro-b.com
Ideal bowel support 299V	*L. plantarum*	Reduz inchaço, gases e desconforto intestinal	www.jarrow.com
Yakult (Yakult)	*L. casei* Shirota	Equilíbra microbiota intestinal	www.yakult.com.br
Actimel (Danone)	*L. casei* Defensis	Regula microbiota intestinal	www.actimel.com
Activia (Danone)	*B. animalis* DN173010	Regula microbiota intestinal	www.activiadanone.com.br

2.4 Seleção de culturas e funcionalidade de BAL

Embora muitas estirpes probióticas de BAL já sejam conhecidas e aplicadas em alimentos fermentados probióticos comerciais em todo o mundo (TAMIME et al., 2005), o mercado de produtos biofuncionais, incluindo os probióticos, está continuamente necessitando de implementação e diversificação dos produtos disponíveis. Para isso, há uma necessidade crescente de identificar novas estirpes biofuncionais, novas estratégias para garantir a sobrevivência dessas culturas e diferentes fontes para o isolamento das estirpes. Estudos recentes mostraram que isolados reconhecidos como probióticos são encontrados também em substratos fermentados não lácteos (RIVERA-ESPINOZA; GALLARDO-NAVARRO, 2010).

A possibilidade de incluir estirpes isoladas de fontes não lácteas em preparações probióticas poderia ampliar o leque de estirpes disponíveis a serem candidatas ao uso como probióticos. Um fator importante que limita a disponibilidade de novas culturas probióticas está ligado aos custos industriais de detecção e caracterização de novos candidatos de estirpes de BAL com propriedades probióticas. Isso levou ao desenvolvimento de diferentes conjuntos de testes simples para a seleção das estirpes *in vitro* (GIRAFFA, 2012).

2.4.1 Critérios para seleção de candidatos a probióticos

Para um probiótico trazer benefício à saúde, alguns critérios devem ser considerados. Devido à amplitude de funções-alvo e de aplicações tecnológicas, a seleção e a avaliação de potenciais candidatos a probióticos exigem uma abordagem abrangente com múltiplos passos. Geralmente, uma caracterização básica inicial de identificação da estirpe e taxonomia é seguida pela seleção daquelas com propriedades funcionais por meio de ensaios validados *in vitro* e

in vivo. Vários aspectos, incluindo origem, sobrevivência às condições impostas pelo ambiente do TGI, funcionalidade e características pró-tecnológicas, devem ser levadas em consideração no processo de seleção de estirpes probióticas (CHASSARD; GRATTEPANCHE; LACROIX, 2011).

2.4.2 Propriedades *in vitro* de importância biológica e tecnológica

Os testes *in vitro*, recomendados pela FAO/WHO (FAO, 2002), são úteis para caracterizar as diferentes estirpes e conhecer o mecanismo do efeito probiótico. Os principais testes *in vitro* atualmente utilizados em estudos de estirpes probióticas incluem resistência abaixo pH, bile, resistência a ácidos, adesão às células epiteliais humanas e linhagens celulares, atividade antimicrobiana contra bactérias potencialmente patogênicas, capacidade de reduzir a adesão de patógenos em superfícies, capacidade de aumentar a resistência da barreira epitelial humana e de linhagens celulares, entre outros. Testes mais sofisticados podem incluir experimentos para verificar o efeito dos probióticos na modulação do sistema imune, por meio da estimulação de células mononucleares de sangue periférico e determinação de citocinas (MARAGKOUDAKIS et al., 2006).

Na maioria dos casos, no entanto, o grande número de estirpes a serem testadas levou à introdução de abordagens de "triagem de subtração", que consistem numa sequência de testes (ex. tolerância à bile, ácidos, etc.) para reduzir progressivamente o número de estirpes candidatas a probiótico. No final das análises, as estirpes que apresentarem o maior número de propriedades funcionais e, concomitantemente, sem traços negativos, são selecionadas. Como exemplo significativo, foi realizado um estudo com 61 isolados de *Lactobacillus* spp. e as bactérias foram submetidas a análises *in vitro* de "triagem de subtração". Todos os isolados foram testados quanto à sua resistência à bile; as

estirpes resistentes foram, então, avaliadas quanto à sua capacidade de sobreviverem a baixos pH. Mais uma vez, aquelas resistentes a pH baixo foram analisadas quanto à possibilidade de exibirem atividades prejudiciais ao hospedeiro (DELGADO et al., 2007). Uma abordagem semelhante foi recentemente aplicada às estirpes de *L. plantarum*, isoladas de produtos lácteos (ZAGO et al., 2011).

No caso dos probioticos que são adicionados a alimentos industrializados, as estirpes candidatas devem sobreviver ao processamento do alimentos e aos estresses biológicos, os quais incluem tolerância à estresse de temperatura, pH, bem como estresse oxidativo e osmótico, além de ataque de bacteriófagos. As estirpes bacterianas que forem utilizadas como *starter* acidificante primária não devem possuir atividade antimicrobiana contra a estirpe probiótica empregada. Culturas probióticas não devem ter efeitos adversos sobre o gosto ou o aroma do produto e não devem aumentar a acidez durante o período de vida útil do produto (GEORGIEVA et al., 2009; MILLS et al., 2011).

2.5 Potencial funcional de bactérias probióticas no TGI

2.5.1 Resistência elétrica transepitelial (TEER)

A homeostase intestinal é essencial para sustentar uma barreira intestinal intacta para a saúde do intestino. Lesões nesta barreira podem resultar em certo número de doenças intestinais, incluindo infecção por patógenos, síndrome do cólon irritável (SCI) e doença de Crohn (SCHULZKE et al., 2009;ZEISSIG et al., 2004). Além disso, uma barreira intestinal comprometida pode levar à exposição das células imunes da mucosa ao conteúdo do lúmen, como antígenos da microbiota intestinal, levando a uma resposta imunológica irregular.

Acredita-se que a homeostase seja mantida por ligações cruzadas entre as células epiteliais e células dendríticas, em especial das mucosas. No entanto, bactérias probióticas e a microbiota intestinal foram reconhecidas como importantes mediadores da função da integridade da barreira intestinal. Resistência elétrica transepitelial, ou TEER, é um método comumente utilizado para determinar a integridade da barreira em linhagens celular epiteliais (SCHNEEBERGER; LYNCH, 2004). As bactérias probióticas, normalmente, promovem um efeito positivo sobre a barreira, em estudos nos quais se empregam linhagens celular *in vitro*, avaliadas por medidas crescentes da TEER (KLINGBERG et al., 2005; NISSEN et al., 2009).

Infecções da linhagem celular do epitelio intestinal de ratos IEC com *E. coli* enteropatogênica resultaram em declínio na TEER, aumentando a permeabilidade epitelial e a disfunção fisiológica. Entretanto, a exposição prévia da linhagem celular IEC com *Lb. acidophilus* vivos limitou os efeitos adversos causados por *E. coli*, fato não observado para células de *Lb. acidophilus* inativadas pelo calor (RESTA-LENERT; BARRETT, 2003). Efeitos positivos adicionais sobre a barreira epitelial, causados por bactérias probióticas, incluem a prevenção da fixação de patógenos aos epitélios, a produção de moléculas de defensina, a atenuação na produção de citocinas pró-inflamatórias e a prevenção de apoptose induzida pela citocina (MENNIGEN; BRUEWER, 2009). Os resultados destes estudos demonstram que as bactérias probióticas podem ter efeito positivo sobre a integridade da barreira, tanto na presença como na ausência de agentes patogênicos.

Estudos realizados em modelos animais confirmaram o aumento da função da barreira de células IEC, devido à atividade de bactérias probióticas (EWASCHUK et al., 2007, 2008; MENNIGEN et al., 2009). Adicionalmente, estudos *in vivo*, juntamente com transcriptômica, continuam a elucidar os

mecanismos exatos pelos quais os microrganismos probióticos aumentam a integridade da barreira epitelial.

Na Figura 1 é apresentado um modelo esquemático de um poço da placa para análises da TEER, simulando o intestino. As células crescem sobre uma membrana microporosa e desenvolvem resistência transepitelial, avaliada entre os compartimentos apical e basolateral. Na Figura 2 observa-se o aparelho *Epithelial voltohmmeter*, EVOM (World Precision Instruments, Inc), empregado para a obtenção dos valores de resistência elétrica transepitelial.

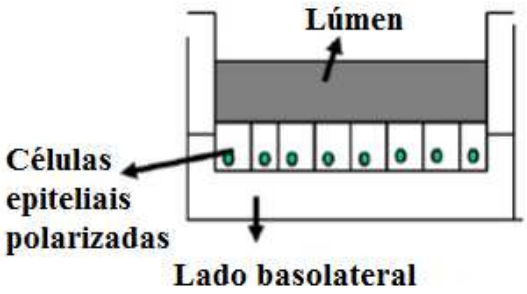

Figura 1 Esquema do modelo utilizado para análises de TEER

Figura 2 Aparelho *Epithelial voltohmmeter*, EVOM (World Precision Instruments, Inc.) utilizado para as avaliações da TEER de linhagens celulares

2.5.2 Atividade antagonica contra patógenos

Estudos *in vitro* relatam capacidade antagonística de culturas probióticas contra bactérias patogênicas (PRIDMORE et al., 2008; RYAN et al.,2009). Bacteriocinas são pequenos peptídeos antimicrobianos, estáveis ao calor, produzidos por várias bactérias, inclusive BAL. Corret al. (2007) demonstraram, *in vivo*, atividade preventiva contra infecções causadas por *Listeria monocytogenes* em ratos, por meio do uso da estirpe *Lb. salivarius* UCC118, que foi capaz de produzir bacteriocina inibindo o crescimento do patógeno *L. monocytogenes.* Métodos adicionais de estudos de atividades antimicrobiana *in vitro* incluem produção de peróxido de hidrogênio (PRIDMORE et al., 2008), ácido láctico (FAYOL-MESSAOUDI et al., 2005), exclusão competitiva (LEE et al., 2003) e na modulação do sistema imunológico (RYAN et al., 2009).

2.5.3 Adesão de células probióticos à células epiteliais intestinal

Um dos critérios mais importantes para a seleção de candidatos a probióticos é a colonização do intestino do hospedeiro, promovendo, assim, seus efeitos biológicos benéficos (FALAGAS; RAFAILIDIS; MAKRIS, 2008; GIBSON; MCCARTNEY; RASTALL, 2005; MYLLYLUOMA et al., 2008; PARKES; SANDERSON; WHELAN, 2009).

A ligação inicial das bactérias é, normalmente, mediada por meio do reconhecimento das porções de carboidrato da superfície da célula intestinal, incluindo glicoproteínas e glicolípidos, conforme apresentado por *Lactobacillus* (CENCIC; LANGERHOC, 2010) e por *Bifidobacterium* (HE et al., 2001). A linhagem celular Caco-2 (Figura 3) é formada por células de adonocarcinoma de cólon humano amplamente utilizadas como modelo *in vitro* para estudos de colonização e mecanismos de interações de células probióticas (CUI et al., 2009;LEE et al., 2009; SCHNABL; FIELD; CLANDININ, 2009; UCHIDA et al., 2009; VISCO et al., 2009).

O conhecimento da capacidade de adesão das bactérias a celulas intestinais é de extrema importância para a seleção de culturas probióticas. Quando aderidas, elas impedem a adesão de bactérias patogênicas e aumentam a função da barreira intestinal, além de serem capazes de estimular o sistema imune do hospedeiro. Em aplicações industriais, em que combinações de estirpes são empregadas, o estudo da capacidade de adesão é imprescindível, já que estirpes individuais podem competir pelo mesmo receptor e, assim, a atividade probiótica pode ser afetada (BOTIC et al., 2007; CORR; HILL; GAHAN, 2009;GUEIMONDE et al., 2007; LI et al., 2008;MOUNTZOURIS et al., 2009).

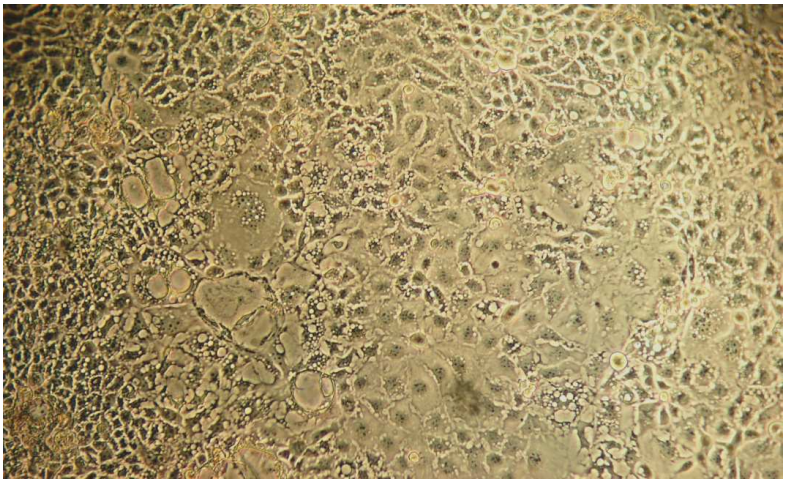

Figura 3 Fotomicrografia de células de adenocarcinoma de cólon humano (Caco-2)

2.6 pH intracelular (pH_i)

O pH_i é um dos fatores mais importantes na manutenção da fisiologia celular dos organismos, já que as enzimas são dependentes de valores de pH para apresentarem sua atividade ótima. Além disso, a concentração de prótons está intrinsecamente envolvida na bioenergética celular. O sistema de força próton-motriz é a fonte central de energia e o gradiente de pH (ΔpH) na membrana celular bacteriana é importante fator para o funcionamento desse sistema (KRULWICH; SACHS; PADAN, 2011).

As células, normalmente, exercem regulação do pH_i em função das condições ambientais. Pesquisas realizadas nesse sentido revelaram que efeitos antibacterianos e antifúngicos de ácidos orgânicos fracos (ARNEBORG; JESPERSEN; JAKOBSEN, 2000; HALM et al., 2004) e nitrito acidificado (MORTENSEN et al., 2008) são atribuídos à acidificação intracelular. Estresses osmóticos induzidos por sacarose e sorbitol (FANG et al., 2004; VINDELOV; ARNEBORG, 2002) e NaCl (MORTENSEN et al., 2007, 2006) também geram

acidificação intracelular de bactérias e leveduras. Adicionalmente, efeitos de compostos antimicrobianos, por exemplo, bacteriocinas e desinfectantes, podem também ser detectados por alterações no pH_i (GAGGIA et al., 2010; KASTBJERG et al., 2009). Além disso, o tipo de acidulante utilizado influencia diretamente a regulação do pH_i. Tanto pH_i quanto fluxos de íons H^+ são mais severamente afetados pelo ácido lático que pelo ácido clorídrico (SHABALA et al., 2006).

Os eucariotos superiores exibem homeostase citoplasmática do pH em valor de pH_i 7,3, com pH extracelular (pH_{ex}) rigorosamente controlado de 7,4. Em contraste, as bactérias neutrófilas podem crescer em valores de pH_{ex}variando de 5,5 a 9,0 e, geralmente, mantêm seus valores de pH_i variando de 7,7 a 7,5. Portanto, a maioria dos neutrófilos tem estratégias para manter o pH_i alcalino em relação ao pH_{ex} em sua extremidade baixa do intervalo de pH de crescimento e pH_i ácido em sua extremidade alta do intervalo de pH (KRULWICH; SACHS; PADAN, 2011).

As BAL são capazes de manter um ΔpH_i sobre uma ampla faixa de valores baixos de pH_{ex}. De acordo com Nannen e Hutkins (1991), em pH_{ex} maior que 5,0, a faixa de pH_i variando de 6,0 a 7,5 é considerada ótima para o crescimento das BAL, enquanto o pH_i 5,0 é considerado crítico. Shabala et al. (2006), ao estudarem a estratégia de *Lb. delbrueckii* subsp. *bulgaricus* para lidar com o estresse ácido, observaram que esta bactéria permite a entrada de prótons após tratamento ácido, de modo que o pH_i segue o pH_{ex} (com baixo ΔpH mantido). Se as células mantiverem o pH_i elevado poderia causar o acúmulo de anions provenientes da fermentação. Portanto, provavelmente, para bactérias fermentativas, é melhor que o pH_i decresça em função do pH_{ex}, resultando em menor acúmulo de ânions no citoplasma.

2.7 Técnicas para avaliação do pH_i

Os primeiros estudos para determinação de pH_i em bactérias baseavam-se na distribuição de ácidos ou bases fracos radioativamente marcados nas células. O método é preciso, porém, apresenta algumas limitações. Neste método, o pH_i é determinado por meio da média de pH_i em uma população de células. O resultado é preciso quando as condições de cultivo são ideais e controladas, porém, muitos estudos são focados nas mudanças do pH_i das células, quando submetidas a condições de cultivo estressantes, como, por exemplo, o ambiente ácido (FOSTER; HALL, 1991; HICKEY; HIRSHFIELD, 1990; O'SULLIVAN; CONDON, 1999). Nestes casos, um número significativo de células pode não ser mais metabolicamente viável (KASHKET, 1985) e, portanto, pode não regular o pH_i da mesma maneira que aquelas metabolicamente viáveis. O valor de pH_i resultante, determinado a partir da mistura de células viáveis e não viáveis, será uma média entre duas subpopulações com diferentes valores de pH_i.

O método de ressonância magnética nuclear utilizando fósforo-31 (RMN 31^P) também é utilizado para a determinação de pH_i em bactérias (FOUCAUD et al., 1995). É um método não invasivo, porém, exige densa população de células e tem tempo de resolução limitado, além de apresentar o mesmo problema dos métodos de distribuição de radioativos (SIEGUMFELDT; ANENBORG, 2011).

Outro princípio para a determinação de pH_i utiliza compostos fluorescentes que apresentam emissões de fluorescências dependentes de pH. Fluoresceína e uma série de derivados de fluoresceína têm intensidades de emissão dependentes de pH quando excitadas a 488 nm, podendo ser utilizadas diretamente para avaliação de pH_i em populações de células utilizando espectrofluorímetro (MAGNI et al., 1999; MOLENAAR; ABEE; KONINGS, 1991). Esta metodologia permite medições rápidas e contínuas, porém, apresenta

as mesmas limitações em relação à análise de subpopulações e, ainda, erros podem ser obtidos por meio de vazamento do corante para a matriz extracelular (BREEUWER et al., 1996). As avaliações mais eficientes de pH_i por meio do uso de fluorescência baseiam-se na relação entre comprimentos de ondas dependentes e independentes de pH.

No método FRIM, o precursor não fluorescente diacetato de carboxifluoresceína é adicionado a uma suspensão de células e entra na célula passivamente. Dentro das células, o composto é hidrolisado por esterases intracelulares, resultando na carboxifluoresceína altamente fluorescente (R = OH). Ao adicionar o grupo succinimidil ao radical (R), o composto pode ligar-se a aminas intracelulares, aumentando a capacidade de permanência no interior da célula, mesmo em células estressadas ou com membrana danificada (Figura 4A). A intensidade de fluorescência após excitação a 488 nm é dependente da concentração do corante e do pH. Para evitar erros decorrentes de intensidades de coloração desiguais, os derivados de fluoresceína são excitados em dois comprimentos de ondas diferentes, no ponto isosbéstico (ponto no qual as curvas todas se juntam e o comprimento de onda da forma ácida e básica tem o mesmo coeficiente de extinção e índices de absorbância iguais) e no máximo de excitação. No ponto isosbéstico, a emissão de fluorescência é independente do pH e a emissão reflete meramente a concentração do corante. Dividindo-se a emissão a partir da excitação no comprimento de onda dependente do pH (488 nm) com a emissão a partir do ponto isosbéstico (435 nm), um valor de índice é obtido, o que reflete somente o valor de pH_i e não a concentração do corante (Figura 4B) (SIEGUMFELDT; ARNEBORG, 2011). Um exemplo da correlação entre o pH e a proporção (490 nm e 440 nm) de diacetato de carboxifluoresceína succinimidil éster (CFDA-SE) é apresentado na Figura 4C.

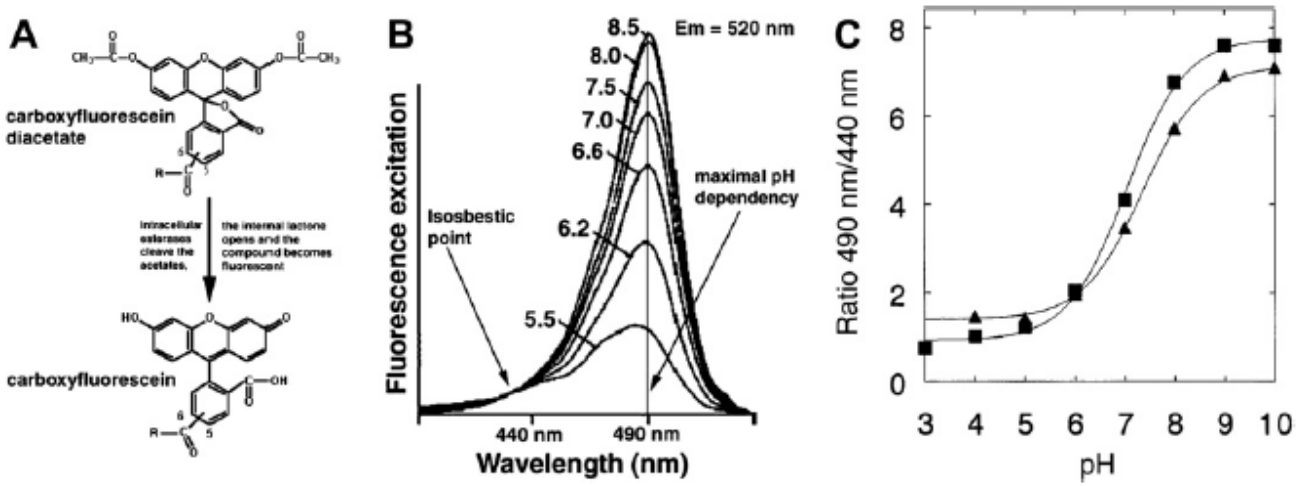

Figura 4(A) Hidrólise intracelular do precursor não fluorescente diacetato de carboxifluoresceína em carboxifluoresceína altamente fluorescente. (B) Curvas relacionando comprimento de onda com taxa de excitação fluorescente em diferentes valores de pH. (C) Relação entre pH e proporção (490 nm e 440 nm) de CFDA-SE em células com pH equilibrado de *Bacillus subtilis* (linha com quadrados), e *Lactococcus lactis* (linha com triângulos). Fonte: Breeuwer et al. (1996)

O método FRIM tem sido utilizado com sucesso para determinações do pH_i de BAL (RECHINGER; SIEGUMFELDT,2002;SHABALA et al., 2006;SIEGUMFELD; RECHINGER; JAKOBSEN, 2000; WU et al., 2012), *Listeria monocytogenes* (KASTBERG et al., 2009; PHAN-THANH; MONTAGNE, 1998), *Escherichia coli* (RIONDET et al., 1997) e também leveduras (ARNEBORG; JESPERSEN; JAKOBSEN, 2000;GULDFELDT; ARNEBORG, 1998; HALM et al., 2004; VALMORRI et al., 2008). Contudo, a faixa de valores de pH_{ex} no qual determinado corante fluorescente pode ser utilizado para avaliação do pH_i é limitada. Os pHs que podem ser determinados por CFDA-SE são de, aproximadamente, 5,0 a 9,0. Embora esta seja uma faixa útil para muitos experimentos, há limitações para experimentos conduzidos em baixos pH, por exemplo, para avaliar alterações de pH_i em estresse ácido. No entanto, este problema pode ser superado utilizando-se sondas com pKa inferior, como, por exemplo, diacetato de carboxi-2′,7′-diclorofluoresceína succinimidil éster (CDCFDA-SE), com a qual os valores de pH_i podem ser determinados em pH abaixo de 4,0 (SHABALA et al., 2006).

FRIM é um método aperfeiçoado em que a determinação da razão do pH_i é utilizada em conjunto com microscópio e análises de imagens de sistemas. Esta técnica é capaz de obter resultados para uma única célula e, portanto, esse método pode resolver o problema na obtenção de pH_i em subpopulações. Além dos resultados obtidos para célula única, FRIM também apresenta vantagens na capacidade de analisar as células em superfícies sólidas e, mesmo, superfícies opacas, como, por exemplo, metal. O primeiro uso da FRIM em bactérias foi para determinação de pH_i no desenvolvimento de esporos de espécies de *Bacillus* (MAGILL et al., 1994, 1996).

2.8 Situações de estresse celular

Devido ao difundido uso de BAL como probióticos, torna-se imprescindível buscar estirpes de BAL resistentes às diversas condições de estresse, já que precisam sobreviver às condições impostas pelo trato digestivo, resistir à competição na microbiota intestinal, colonizar a mucosa digestiva ou urogenital e expressar funções específicas em condições desfavoráveis para o crescimento (por exemplo, durante fase estacionária). É essencial conhecer não só quais condições são desfavoráveis ou prejudiciais para a vida das BAL, mas também quais mecanismos permitem sua sobrevivência e atividades metabólicas sob condições estressantes (ANGELIS; GOBBETTI, 2004; GUCHTE et al., 2002; MILLS et al., 2011; ZANG et al., 2012).

As células bacterianas são naturalmente equipadas com uma variedade de mecanismos de defesa para aumentar a sobrevivência em ambientes hostis (MILLS et al., 2011). Dentre estes mecanismos, incluem-se proteínas chaperonas que auxiliam na conformação das proteínas deformadas, proteases que degradam proteínas irreversivelmente danificadas, sistemas de transporte para manter a osmolaridade correta, catalases e superóxido dismutase para combater espécies reativas de oxigênio, bem como bombas de prótons, descarboxilases e transportadores para combater a queda do pH_i (ANGELIS; GOBBETTI, 2004; GUCHTE et al., 2002; MILLS et al., 2011). Na Figura 5 estão esquematizadas as principais respostas celulares ao estresse causado por ácidos e bile.

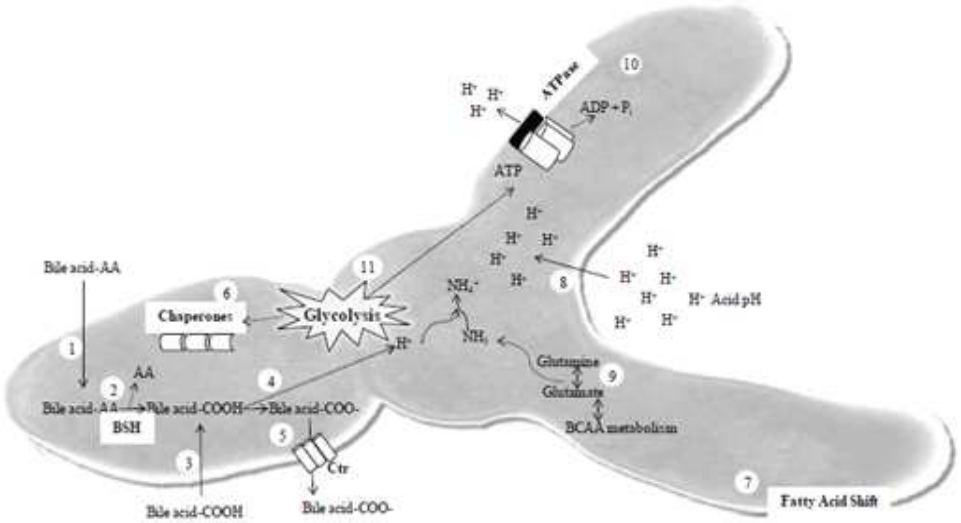

Figura 5 Representação esquemática das principais respostas ao estresse em pH ácido e bile em bifidobactérias. (1) Os ácidos biliares conjugados entram no citoplasma bacterianoe são clivados pela BSH (sal biliar hidrolase) (2) liberando aminoácido e radical de ácido biliar desconjugado. (3) Ácidos biliares desconjugados também podem entrar na célula por difusão passiva e se tornam desprotonados (4). (5)Sais biliares ionizados são impermeáveis e são excretados pela ação de certos transportadores, por exemplo Ctr (transportador colato) de *Bifidobacterium longum*. (6) Aumento da síntese de chaperonas moleculares e mudança na composição de ácidos graxos da membrana celular podem ocorrer (7). Exposição ao pH ácido ou desprotonação de sais biliares resulta na acidificação do citoplasma (8). Isso pode ser contrabalançado pela produção de amônia a partir da desaminação de glutamina (9) ou pelo bombeamento prótons da célula pelo sistema F_0F_1-ATPase (10) .ATPs requeridos para a realização desses sistemas são gerados através da glicólise (11).Fonte: Mills et al. (2011)

2.8.1 Estresse ácido

Ambientes ácidos são importantes causas de estresse para BAL. As estirpes probióticas são expostas a estresse extremo de ácidos quando chegam ao estômago, onde o ácido hidroclorídrico está presente. Em resposta ao estresse ácido, BAL empregam numerosos mecanismos para sobreviver ao baixo pH, incluindo aumento da atividade e da quantidade de F_0-F_1-ATPase (LORCA; VALDEZ, 2001; MATSUMOTO; OHISHI; BENNO, 2004), do sistema de reparo de DNA induzido por baixo pH (LEBEER; VANDERLEYDEN;DE KEERSMAECKER, 2008), manutenção da homeostase do pH_i (HUTKINS; NANNEN, 1993; SIEGUMFELDT; RECHINGER; JAKOBSEN, 2000) e alterações no envelope celular (ZHANG; ROCK, 2008) (Figura 5). O sistema de transporte de prótons ATPase é o de maior importância para bactérias fermentativas (HUTKINS; NANNEN, 1993). Esta enzima foi caracterizada como *Lb. casei* e *Lb. Plantarum*, e sua atividade ótima foi encontrada em valores de pH de 5,0-5,5 (BENDER; MARQUIS, 1987; NANNEN; HUTKINS, 1991), valores menores que aqueles (pH 7,0-7,5) encontrados para *S. thermophilus* e *Lc. lactis* subsp. *lactis* (NANNEN; HUTKINS, 1991). A permeabilidade da membrana plasmática a prótons também contribui para a regulação do pH_i (BENDER; MARQUIS, 1987).

Com exceção de algumas espécies dos gêneros *Lactobacillus*, *Leuconostoc* e *Oenococcus*, BAL são neutrófilas. Os efeitos do estresse ácido sobre a fisiologia bacteriananão são conhecidos em detalhe. No entanto, está bem estabelecido que ácidos podem se difundir passivamente através da membrana da célula e, após a entrada no citoplasma, rapidamente dissociam-se em prótons e derivados carregados que se tornam impermeáveis à membrana celular (PRESSER; RATKOWSKY; ROSS, 1997). O acúmulo de prótons intracelulares pode baixar o pH_i e, assim, afeta o ΔpH transmembrânico, que

contribui para a força próton-motriz, utilizada como fonte de energia em numerosos processos de transporte transmembrânico. A acidificação interna também reduz a atividade de enzimas sensíveis a ácido e danifica proteínas e DNA. O acúmulo no citoplasma das unidades aniônicas dos ácidos orgânicos dissociados provoca efeito prejudicial sobre a fisiologia celular (PRESSER; RATKOWSKY; ROSS, 1997), provalvelmente por meio de interações quelantes com elementos essenciais.

Em BAL, a tolerância a ácidos (AT) aumenta em pelo menos dois distintos estados fisiológicos: (1) durante o crescimento logarítmico, a resposta adaptativa, referida como L-ATR, pode ser induzida pela incubação em concentrações não letais de pH ácidos; (2) após entrada na fase estacionária, AT aumenta como resultado da indução da resposta ao estresse geral (GSR) (HARTKE et al., 1996). Este último é, geralmente, uma resposta independente do pH_{ex}, embora para *Lb.acidophilus* CRL639 o pH_{ex} baixo seja necessário (LORCA; VALDEZ, 2001). Não se sabe se estas respostas são independentes ou coincidentes.

2.8.2 Estresse causado pela bile

Como já descrito anteriormente, além da resistência a baixo pH, adesão ao tecido epitelial intestinal e produção de substâncias antimicrobianas, a resistência à toxicidade da bile é um dos critérios utilizados para a seleção de linhagens probióticas (DELGADOet al., 2007; GILLILAND; STALEY; BUSH, 1984). Portanto, a maior parte dos trabalhos sobre tolerância bacteriana à bile é realizada por pesquisadores que buscam estirpes probióticas. No entanto, uma vez que resistência à bile é apenas um dos muitos critérios utilizados para selecionar as linhagens, as análises não são realizadas profundamente e,

simplesmente, comparam a capacidade das estirpes de sobreviver na presença de bile (BEGLEY; GAHAN; HILL, 2005).

Acredita-se que a desconjugação de sais biliares seja um mecanismo de desintoxicação e as enzimas BSH desempenham papel importante na tolerância à bile e, consequentemente, na sobrevivência no trato gastrintestinal.

Em estudos realizados por três grupos independentes usando tipos selvagens e mutantes para a atividade de BSH estabeleceu-se uma ligação entre a hidrólise de sais biliares e tolerância à bile (DUSSURGET et al., 2002; GRILL et al., 2000; SMET et al., 1995). O mutante *Lb. Amylovorus*, apresentando atividade parcial de BSH, apresentou decréscimo na taxa de crescimento na presença de sais biliares (GRILL et al., 2000). Dussurget et al. (2002) demonstraram que a deleção do gene para síntese da enzima BSH em *L. monocytogenes* diminuiu as concentrações inibitórias mínimas para bile e sais biliares. Por fim, a estirpe *Lb. plantarum* mutante para BSH, obtida por Smet et al. (1995), apresentou inibição fortemente dependente de pH. Os autores sugerem que, como a forma protonada (não dissociada) dos sais biliares parece apresentar toxicidade através de acidificação intracelular de maneira similar aos ácidos orgânicos, as células positivas para BSH podem se proteger por meio da formação de homólogos não conjugados fracos. Isso pode ajudar a evitar a queda do pH por meio da recaptura e da exportação do próton cotransportado. Em conjunto, estes três estudos com mutantes BSH sugerem importante papel para a enzima BSH na tolerância à bile e, particularmente, a tolerância a sais biliares glicoconjugados (BEGLEY; GAHAN; HILL, 2005).

Hidrolases de sais biliares convertem os sais biliares protonados e conjugados de que entram na célula em seus homólogos fracos desconjugados que podem recapturar prótonsco-transportados, evitando, assim, gasto excessivo de ATP para manter a homeostase do pH. Essa energia pode ser gasta em células

com atividade BSH negativa, uma vez que não pode formar sais biliares não conjugados que capturam os prótons (Figura 5).

3 CONSIDERAÇÕES FINAIS

O grande interesse em buscar alternativas alimentares associadas à promoção de saúde vem aumentando de maneira considerável, ultimamente. Portanto, o estudo de características probióticas dos microrganismos tem se tornado de grande importância.

Probióticos são microrganismos amplamente utilizados como suplementos alimentares ou adicionados a produtos alimentícios, no intuito de gerar benefícios à saúde. Alimentos e/ou processos fermentativos para a produção de alimentos são considerados ricas fontes de microrganismos cujas atividades funcionais ainda são pouco conhecidas. Visto que estirpes microbianas podem apresentar diferentes propriedades probióticas, o estudo de diferentes estirpes isoladas de diferentes produtos/processos torna-se imprescindível. A atuação do probiótico, geralmente, ocorre no intestino, portanto, é necessário que o candidato a probiótico sobreviva às severas condições impostas pelo TGI. Avaliações da homeostase do pH_i são uma maneira eficiente de avaliar as condições fisiológicas celular, sendo possível acompanhar este comportamento em uma única célula, por meio do método de FRIM. Esta metodologia, associada às condições simuladas do TGI, possibilita o acompanhamento do estado fisiológico de células microbianas individualmente, durante a passagem no ambiente do TGI.

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SEGUNDA PARTE – ARTIGOS

ARTIGO 1

STRAIN-SPECIFIC PROBIOTICS PROPERTIES OF *Lactobacillus fermentum*, *L. plantarum* and *L. brevis* ISOLATES FROM BRAZILIAN FOOD PRODUCTS

Artigo redigido conforme norma da Revista à Food Microbiology, sujeito a modificações conforme sugestões do conselho editorial da revista

Abstract

A total of 234 lactic acid bacteria (LAB) isolates from Brazilian food products were initially screened for their ability to survive at pH 2.0. Fifty one of the isolates survived and were selected. They were identified using 16S rRNA gene sequencing as *Lactobacillus fermentum* (34 isolates), *Lactobacillus plantarum* (10)and *Lactobacillus brevis* (7). Based on being either highly tolerant to bile, showing a ability for auto-aggregation and/or hydrophobic properties, one *L. fermentum* (CH58), three *L. plantarum* (CH3, CH41 and SAU96) and two *L. brevis* (SAU105 and FFC199) were selected. The highest co-aggregation ability with *Escherichia coli* was observed to *L.plantarum* CH41. *L.brevis* SAU105 and FFC199 and *L. fermentum* CH58 exhibited antagonistic activity towards the pathogens *Listeria monocytogenes* and *Staphylococcus aureus*. *L. plantarum* CH3 and CH41 and *L. brevis* FFC199 showed adhesion ability to Caco-2 cells (1.6, 1.1 and 0.9 %, respectively) similar to the commercial probiotic, *L. rhamnosus* GG (1,5%) . They were able to increase the transepithelial electrical resistance (TEER) of Caco-2 cells over 24 h ($p<0.05$). The present work showed that the probiotic characteristics were strain-specific and that the isolates *L. plantarum* CH3 and CH41 (cocoa) and *L. brevis* FFC199 (cauim) exhibited potential probiotics properties. Thus, it was possible to associate these products with functional characteristics.

Keywords: LAB, probiotic, functional properties, rep-PCR, adhesion, TEER.

Resumo

Um total de 235 isolados de bactérias do ácido lático (BAL) provenientes de produtos alimentícios brasileiros foi inicialmente avaliado quanto à habilidade de sobreviver em pH 2,0. Cinquenta e um isolados foram capazes de sobreviver, sendo, então, selecionados e identificados, por meio do sequenciamento do gene 16S rRNA, como *Lactobacillus fermentum* (34 isolados), *Lactobacillus plantarum* (10) e *Lactobacillus brevis* (7). Baseado no fato de serem tanto altamente tolerantes à bile quanto possuírem capacidade de autoagregação e/ou propriedades hidrofóbicas, um *L. fermentum* (CH58), três *L. plantarum* (CH3, CH41 e SAU96) e dois *L. brevis* (SAU105 e FFC199) foram selecionados. A maior capacidade de coagregação com *Escherichia coli* foi observada para *L.plantarum* CH41. *L.brevis* SAU105 e FFC199 e *L. fermentum* CH58 apresentaram atividade antagônica em relação aos patógenos *Listeria monocytogenes* e *Staphylococcus aureus*. *L. plantarum* CH3 e CH41 e *L. brevis* FFC199 mostraram capacidade de adesão às células Caco-2 (1,6; 1,1% e 0,9%, respectivamente), semelhante ao probiótico comercial, *L. rhamnosus* GG (1,5%). Estes foram também capazes de aumentar a resistência elétrica da barreira transepitelial (TEER) das células Caco-2, durante 24 horas ($p < 0,05$). No presente trabalho mostrou-se que as características probióticas foram estirpe-específicas e que os isolados de *L. plantarum* CH3 e CH41 (cacau) e *L. brevis* FFC199 (cauim) exibiram potenciais propriedades probióticas. Foi, então, possível agregar valor nutricional a estes produtos.

Palavras-chaves: BAL, probióticos, propriedades funcionais, rep-PCR, adesão, TEER.

1. Introduction

Lactic acid bacteria (LAB) have been used as food supplements, and are highly valued for their probiotic properties. Many probiotic isolates, such as *L. acidophilus* LA1, *L. rhamnosus* GG, *L. plantarum* Lp01, *L. fermentum* RC-14 are already widely used and produced on an industrial scale (Giraffa et al., 2010). However, a large number of potentially probiotic microorganisms present in different kinds of food products continue unknown. Spontaneously fermented foods may constitute a reservoir for new LAB spp. strains with potential probiotic characteristics (Mathara et al. 2008; Taked et al. 2011). Several naturally fermented products from Brazil harbor lactic acid bacteria. These include Cauim (kawi) which is a non-alcoholic beverage from various substrates produced by Brazilian Indians, with *Lactobacillus* spp. being the predominant bacteria found in this product (Almeida et al., 2007; Ramos et al., 2010, 2011). Another traditional process in Brazil involving LAB is the cocoa fermentation, which is a key step in the technological transformation of cocoa into chocolate (Pereira et al., 2012). Finally, an industrial product consumed in Brazil and worldwide is fresh sausage. It is freshly prepared and not fermented. However, it constitutes a reservoir of LAB (Ammor & Mayo, 2007; Dias et al., 2013). Although the LAB microbiota of the above mentioned products have previously been identified (Almeida et al., 2007; Pereira et al., 2012; Ramos et al., 2010, 2011), their probiotic potential has not been characterized.

Giraffa et al. (2010) described that in order for a probiotic to be of benefit to human health, it must survive passage through the upper GIT (gastro intestinal tract) and be able to function in the gut environment. The functional requirements of probiotics include tolerance to acid and bile, adherence to epithelial surfaces and antagonistic activity toward intestinal pathogens. Auto-aggregation of probiotic strains is thought to influence the adhesion capacity of

the bacteria cells to intestinal epithelial cells, and co-aggregation with pathogens may prevent colonization of the latter in the gut (Del Re et al. 2000). Bacterial adhesion is initially based on non-specific physical-chemical interactions between two surfaces and the physical and chemical characteristics of the bacterial cell surface depend on surface hydrophobicity (Kotzamanidis et al., 2010; Kos et al., 2003).

Mammalian epithelial cells have been used as *in vitro* models for studying the adhesion of probiotic bacteria to the gastrointestinal (GI) tract (Anderson et al., 2010; Klingberg et al., 2005a) and to determine the effect on the strength of the epithelium barrier by measurements of transepithelial electrical resistance (TEER) (Klingberg et al., 2005b). Treatment with probiotic bacteria may prevent or reverse increased permeability of the epithelium and act antagonistically towards pathogens (Klingberg et al., 2005b). Measurements of the TEER of polarized monolayers of epithelial cells are therefore seen as a useful method of providing information about potential probiotic effect of microorganisms (Anderson et al., 2010; Klingberg et al., 2005b).

The objective of this study was to identify to species level and evaluate the probiotic properties of LAB isolated from Brazilian food products. They were characterized by phenotypic tests, rep-PCR and identified by 16S rRNA gene sequencing. Probiotic properties were evaluated by tolerance to low pH and bile, surfaces properties (aggregation, co-aggregation and hydrophobicity), antagonistic activity towards pathogenic bacteria and by assays employing the Caco-2 cell line (adhesion and TEER).

2. Material and Methods

2.1. Bacterial isolates and culture conditions

A total of 234 LAB isolates belonging to the microbial collection of the Microbial Physiology Laboratory/Department of Biology /Federal University of Lavras (UFLA), Brazil, and isolated from cocoa fermentation (99 isolates) (Pereira et al., 2012), industrial sausage (91 isolates) (Nobre et al., 2013) and fermented food cauim (44 isolates) (Ramos et al., 2010) were initially employed in this study. The samples were previously processed (Pereira et al., 2012; Ramos et al., 2010). However, the particular isolates employed in the present work had not been fully identified. They were presumptively identified as LAB by culturing on Man Rogosa Sharpe (MRS, Merck, Darmstadt, Germany) agar and examining for colony and cell appearance, catalase activity, Gram stain, motility and the production of CO_2 from glucose in MRS broth with a Durham tube.

The LAB isolates were cultured in MRS broth at 37 °C for 24 h and stored at -80 °C with 20% (v/v) glycerol. The pathogenic strains *Escherichia coli* JM109, *Salmonella* Typhimurium 224/87, *Staphylococcus aureus*, *Listeria monocytogenes* EGDe, *L. monocytogenes* LO28 and *L. monocytogenes* 4446 obtained from the culture collection of the Food Science Department, Faculty of Science, Copenhagen University, were employed in the antagonistic (section *2.3.5*) and co-aggregation (*E. coli* JM109) (section *2.3.4*) assays. They were grown in Brain Heart Infusion (BHI; Oxoid, Roskilde, Denmark) broth, 37 °C for 24 h and stored as described above.

2.2. Characterization and identification

2.2.1. Rep-PCR

The 51 selected isolates were genotypically characterized by rep-PCR. Genomic DNAs were extracted using Instagene (Bio-Rad, Deutschland) following the instructions of the manufacturer. Fingerprints of genomic DNA were obtained with PCR amplification of repetitive bacterial DNA elements (rep-PCR) using the $(GTG)_5$ primer as described by Nielsen et al., 2007. Cluster analysis were performed using BioNumerics software (version 4.50), similarities were calculated using DICE correlation coefficient and UPGMA algorithm as previously described (Parkouda et al., 2010).

2.2.2. Identification to species level by 16S rRNA gene sequencing and phenotypic tests

Representatives of each group obtained by rep-PCR were selected for16S rRNA gene sequencing gene using the primers 7F (5' AGA GTT TGA TYM TGG CTC AG 3') and 1510R (5' ACG GYT ACC TTG TTA CGA CTT 3') according to Padonou et al. (2009). Amplified PCR products were sent to sequencing at Macrogen (Netherlands). Searches in EzTaxon server (http://www.eztaxon.org/; Chun et al., 2007) were performed to determine the closest known relatives of the partial 16S rRNA gene sequences that were obtained. Phenotypic assays were performed to confirm the identification. Fermentations of cellobiose, melezitose, gluconate, glycerol and xylose, and growth at 45°C were evaluated as recommended in The Prokaryotes (Hammes & Hertel, 2006).

2.3 Screening for probiotic properties

2.3.1. Tolerance to pH 2.0

The 234 isolates were subjected to a pH 2.0 tolerance assay in order to select the resistant isolates for further studies. LAB cells cultivated in MRS broth (Merck, Darmstadt, Germany) at 37 °C for 24h were centrifuged (5000 rpm for 5 min at 24 °C) and washed two times in 0.1% w/v peptone water pH 7.0. The cell cultures (optical density of 0.2 at 600 nm)in peptone water corresponding to approximately 10^8 cell / mL were centrifuged and re-suspended in MRS broth (Merck, Darmstadt, Germany) with pH adjusted to 2.0 using 1N HCl and incubated for 3 h at 37°C. Samples (10 µL) were obtained at time 0 and after 3 h and added to in MRS agar. Tolerance to pH 2.0 was indicated by subsequent growth on MRS agar plates after 48 h of incubation at 37 °C.

2.3.2. Determination of bile tolerance

To study the effect of bile on the growth rate of the 51 acid tolerant LAB isolates (*sec. 2.3.1*), a method described by Guo et al. (2009) was utilized. Tolerance to bile was evaluated based on the time required to increase the absorbance at 600 nm by 0.3 units in MRS broth with and without 0.3% (w/v) oxgall (Sigma Chemical Co., St Louis, MO). The difference in time (hours) to obtain 0.3 units between the measurements of the culture media with and without bile was considered as the adaptation time (AT) of the cells to adapt to media containing bile. The experiment was performed in duplicate.

2.3.3. Cell surface hydrophobicity

Bacterial cell surface hydrophobicity was assessed for the 51 acid tolerant isolates (*sec. 2.3.1*) by measuring microbial adhesion to hydrocarbons (MATH) as described by Kotzamanidis et al. (2010). The assay was performed in duplicate and repeated twice.

2.3.4. Auto-aggregation and co-aggregation assay

Auto-aggregation assays were performed according to Kos et al. (2003). The 51 acid tolerant isolates were grown for 18 h at 37 °C in MRS broth medium (static). The cells were harvested by centrifugation at 5000 g for 15 min, washed twice and re-suspended in PBS to give viable counts of approximately 10^8 CFU / mL (optical density of 0.2 at 600 nm). Cell suspensions (4 mL) were mixed by vortexing for 10 s and auto-aggregation was determined after 5 h of incubation at 37 °C. Every hour, 0.1 mL of the upper suspension was transferred to another tube with 3.9 mL of PBS and the absorbance (A) was measured at 600 nm. The auto-aggregation percentage is expressed as: $1- (A_t/A_0) \times 100$, where A_t represents the absorbance at time t = 1, 2, 3, 4 or 5 h and A_0 the absorbance at $t = 0$.

Co-aggregation assay was performed with the 6 selected LAB isolates. The method for preparing the cell suspensions for co-aggregation was the same as for the auto-aggregation assay. Equal volumes (2 mL) of each cell suspension were mixed together in pairs by vortexing for 10 s. Control tubes were set up at the same time, containing 4 mL of each bacterial suspension on its own. The absorbencies (*A*) at 600 nm of the suspensions were measured after mixing, and after 5 h of incubation at room temperature. Samples were taken in the same

way as in the auto-aggregation assay. The percentage of co-aggregation was calculated using the equation of Handley et al. (1987):

$$\text{Co-aggregation (\%)} = (((Ax + Ay)/2) - A(x+y) / (Ax + Ay)/2) \times 100$$

Where x and y represent each of the two isolates in the control tubes, and (x+y) the mixture. The aggregation and co-aggregation assays were performed in duplicate and repeated twice.

2.3.5. Antagonism towards the Escherichia coli, Salmonella Typhimurium, Staphylococcus aureus and Listeria monocytogenes species

The antagonism towards various bacterial pathogens was evaluated according to Zago et al. (2011) with some modifications. Overnight cultures grown in Brain Heart Infusion (BHI; Oxoid, Roskilde, Denmark) broth were inoculated (2% v/v) in BHI agar (melted and then cooled down to temperature between 45-50 °C). After vigorous homogenization, cell suspensions were poured into Petri dishes. Wells (5 mm) were made on each agar plate. Overnight cultures of the 6 selected LAB isolates were centrifuged and the supernatants were recovered. The supernatant was filter-sterilized (0.45 mm, Millipore, Cork, Ireland) and pH was adjusted to 7.0, aliquots of 30 µl of each supernatant were added to the wells. Petri dishes were incubated for 24 h at 37 °C and the diameters of the halos of inhibition were recorded in mm. The assay was performed in triplicate.

2.3.6. Growth and maintenance of Caco-2 cells

The Caco-2 cells (DSMZ, Braunschweig, Germany) were grown in Modified Eagles Medium (MEM) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS; Lonza, Basel, Switzerland), 1x Non Essential Amino Acids (NEAA), and 0.1 mg / mL gentamicin. All solutions were obtained from Invitrogen, Gibco (Naerum,Denmark). The cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

2.3.7. Adhesion capacity to Caco-2 cell line

Bacterial adhesion capacity to the human colon adenocarcinoma cell line Caco-2 was investigated for the 6 selected LAB isolates. The Caco-2 cells were sub-cultivated (2 x 10^5 cell / mL) in 24 well tissue culture plates (Nunc, Roskilde, Denmark) and grown at 37 °C in a humidified atmosphere of 5% CO_2 for 21 days to obtain differentiation (Riedel et al., 2006) in cell media without antibiotics. The adhesion assay was performed as previously described (Klingberg et al., 2005a). Experiments were performed with duplicate determinations and repeated three times. The bacterium *L. rhamnosus* GG (LGG; Valio Ltd, Helsinki, Finland) was employed as a reference strain.

2.3.8. Transepithelial electrical resistance as measurements of epithelial barrier function

The bacterial effect on the epithelial barrier was evaluated by measurement of TEER using the Millicell Electrical Resistance System (Millipore, Bedford, MA) as previously described (Klingberg et al., 2005b). To obtain polarized monolayers, Caco-2 cells were seeded onto Transwell filter

inserts (0.4 µm pore size, 12 mm inside diameter, polycarbonate; Corning Incorporated, Corning, NY) at a concentration of $2x10^5$ cells / mL and cultivated for 21 days. Overnight cultures of the 6 selected isolates were suspended in cell growth medium without antibiotics. The bacterial suspension (500 µl) was added to the apical compartment at 10^6 CFU / ml and incubated at 37 °C in a humidified atmosphere of 5% CO_2. TEER was measured before the addition of the bacteria (time zero) and then at various time intervals and expressed as the ratio of TEER at time t in relation to the initial value (at time zero) for each series. The net value of the TEER was corrected for background resistance by subtracting the contribution of cell free filter and the medium (150 ohms). The TEER of monolayers without added bacteria represented the control for each experiment. Experiments were performed with triplicate determinations and repeated twice. The probiotic bacterium LGG was also employed as a reference strain.

2.4. Statistical analyses

Analyses of the variance and the Scott–Knott test were performed with SISVAR 5.1 software (Ferreira, 2008). Principal component analyses (PCA) were performed using the XLSTAT 7.5.2 software (Addinsoft's, New York, NY, USA).

2.5 Nucleotide accession numbers

The nucleotide sequences determined in this study have been assigned to GenBank Accession numbers JX845637 - JX845650.

3. Results

3.1. Selection of isolates for further characterization

Tolerance to low pH of 2.0 in MRS broth was chosen as selection criteria for isolates to be included in further experiments. Amongst the 234 LAB investigated, a total of 51 isolates were tolerant to pH 2.0: 43 from cocoa fermentation, 6 from industrial sausage and 2 from cauim.

3.2 Identification of LAB to species level

The 51 acid tolerant LAB isolates were clustered according to their rep-PCR profiles into 3 groupsat a similarity level of 60%(Fig.1). According to the grouping by rep-PCR, 14 strains were selected and sequenced. Based on 16S rRNA gene sequence analysis against sequences in the EzTaxon server, the isolates of group 1, 2 and 3 were identified as *Lactobacillus fermentum* (7 isolates), *Lactobacillus plantarum* (4) and *Lactobacillus brevis* (3), respectively. The similarity values found ranged from 99.42 to 100 % for *L. fermentum*, 99.92 to 100 % for *L. plantarum* and 99.92 to 100 % for *L. brevis*. The isolates identified as *L. plantarum* by 16S rRNA gene analysis were able to ferment cellobiose, gluconate and melezitose, but not xylose and glycerol. In comparison, isolates identified as *L. fermentum* were able to ferment xylose and gluconate but not cellobiose, melezitose and glycerol. The isolates identified as *L. brevis* were able to ferment gluconate and xylose, but not cellobiose, melezitose and glycerol. All isolates grew at 45 °C, except the *L. fermentum* isolates. The phenotypic results confirm the identity obtained by 16S rRNA gene sequencing.

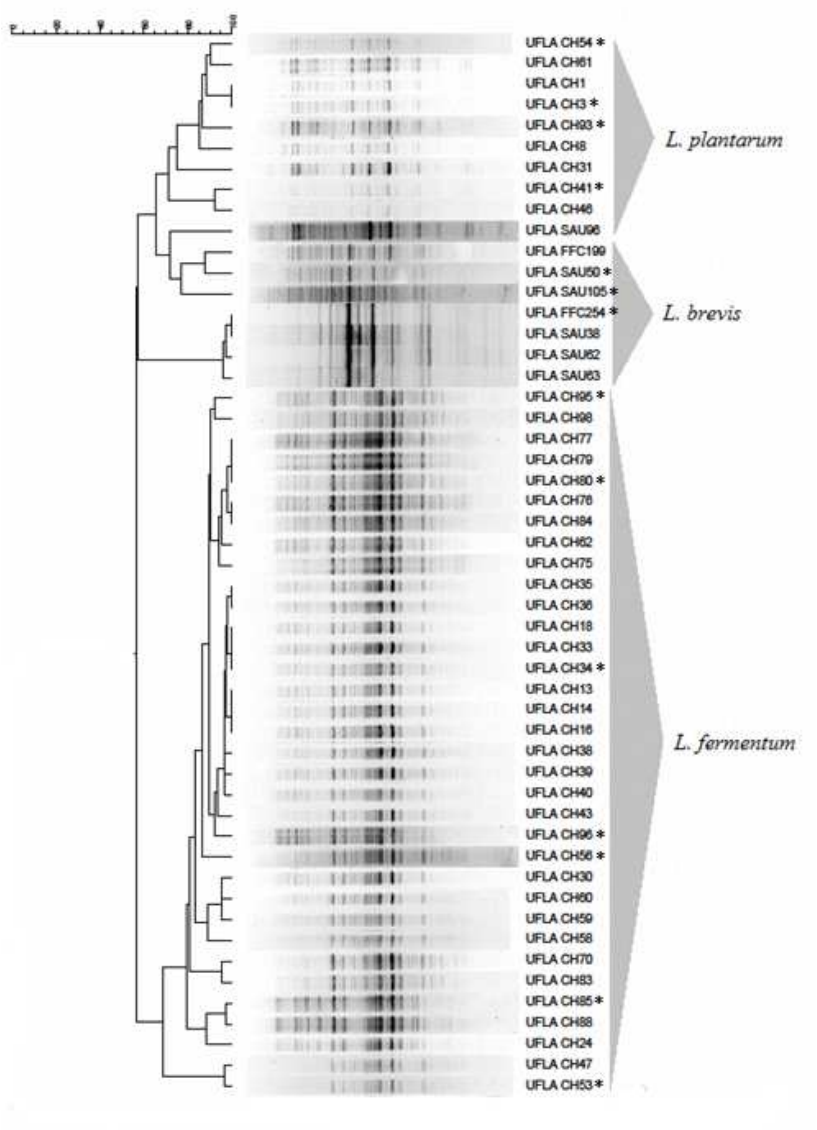

Figure 1 Dendrogram generated after cluster analysis of rep-PCR fingerprints of the *Lactobacillus* strains. The reference codes indicate the source of LAB: CH = cocoa fermentation, SAU = sausageand FFC = fermented food cauim. Stars indicate isolates selected to sequence analysis

3.3. Bile tolerance, hydrophobicity and auto-aggregation properties

The 51 LAB isolates selected by tolerance to pH 2.0 and identified to species level were evaluated according to their bile tolerance and surface properties (hydrophobicity and auto-aggregation assays). The results obtained from these assays were subject to PCA analysis (Fig. 2). The first (PC1) and the second (PC2) principal components explain 41.9 and 22.2 % of the total variance, respectively.

All of the 51 LAB isolates were able to tolerate bile salts at 0.3 % (w/v) (oxgall) for 3 hours. However, the growth and time of adaptation in the bile media was strain dependent (data not shown). Isolates *L. plantarum* CH3, CH41 and *L. brevis* FFC199 had the shortest adaptation time ($p<0.05$) in the bile media of approximately one hour, while *L. brevis* SAU62 and SAU38, had the longest adaptation time of 4 hours. Regarding surface properties, excepted for *L. brevis* SAU105 which was highly hydrophobic (hydrophobicity value of 61.0 %) all other isolates showed to be non-hydrophobic ($H \leq 1.5\%$). Furthermore, *L. plantarum* SAU96 and *L. fermentum* CH58 showed the highest ability for auto-aggregation (61.9 and 55.1 %, respectively, $p<0.05$, Table 1) as compared to the other isolates showing moderate auto-aggregation (12.1-21.0 %). As observed in the PCA (Fig. 2), there were no correlations between product type or species and grouping according to the investigated properties. On the negative side of PCA 1, the isolates were not correlated with high values of adaptation time in media with bile, in other words they had short time to adapt to media with bile. On the negative side of PCA 1 and PCA 2, the isolates were correlated with auto-aggregation ability, while on the positive side of PCA 1 and PCA 2, the isolates were correlated with percentage of hydrophobicity, showing the isolate *L. brevis* SAU105 highly related to hydrophobicity propertie.

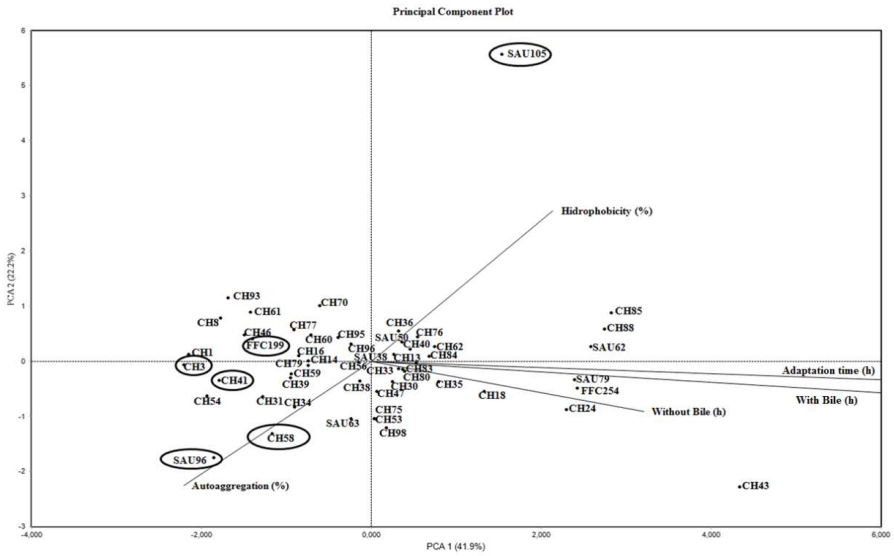

Figure 2 Principal Component Analysis (PCA) of functional proprieties as tolerance to bile (growth in presence and absence of bile, as well the time of adaptation) and surface pattern (hydrophobicity and auto-aggregation) for the 51 *Lactobacillus* isolates. Isolates highlighted were the selected strains

Table 1 Tolerance of selected *Lactobacillus* strains to bile salts, auto-aggregation, and hydrophobicity. AT = adaptation time. The reference codes indicate the source of LAB: CH = cocoa fermentation, SAU = sausageand FFC = fermented food cauim

Isolate	**Time required to increase A620 nm 0.3units**			**Auto-aggregation (%)**	**Hydrophobicy (%)**
	MRS (h)	**MRS+ bile (h)**	**AT (h)**		
L. plantarum CH3	2.94 ± 0.04^{a}	3.71 ± 0.08^{a}	0.76 ± 0.12^{a}	20.94 ± 1.65^{b}	0.83 ± 0.05^{a}
L. plantarum CH41	4.01 ± 0.03^{c}	4.45 ± 0.10^{b}	0.43 ± 0.06^{a}	18.08 ± 0.34^{b}	0.00 ± 0.00^{a}
L. fermentum CH58	4.46 ± 0.07^{d}	6.07 ± 0.17^{d}	1.61 ± 0.24^{b}	55.61 ± 1.82^{c}	1.48 ± 0.64^{a}
L. brevis FFC199	3.71 ± 0.10^{b}	4.74 ± 0.01^{b}	1.03 ± 0.09^{a}	16.31 ± 0.61^{b}	0.26 ± 0.03^{a}
L. plantarum SAU96	3.54 ± 0.15^{b}	5.23 ± 0.26^{c}	1.69 ± 0.11^{b}	61.89 ± 0.44^{c}	0.00 ± 0.00^{a}
L. brevis SAU105	4.56 ± 0.26^{d}	7.41 ± 0.08^{e}	2.85 ± 0.35^{c}	12.17 ± 0.24^{a}	60.98 ± 2.84^{b}

Presented values are means of triplicate determinations; ± indicates standard deviations from the mean. Mean values (± standard deviation) within the same column followed by different superscript letters differ significantly ($P < 0.05$) by Scott–Knott test

3.4. LAB pathogen interactions

Based on bile tolerance and surface properties(auto-aggregation and hydrophobicity) (Fig. 2) the following isolates: *L. plantarum* CH3, CH41 and SAU96, *L. fermentum* CH58 and *L. brevis* SAU105 and FFC199 (highlighted in the PCA, Fig.2) were selected for co-aggregation experiments with *E. coli* JM109 and screened for antagonistic activity against relevant pathogens (Table 2). Further, these selected isolates were subjected to adhesion and TEER assays with Caco-2 cell line. Co-aggregation values ranged between 1.1 % (*L. brevis* FFC199) and 30.7 % (*L. plantarum* CH41). The *L. plantarum* SAU96, *L. fermentum* CH58 and *L. brevis* SAU105 and FFC199 isolates showed antagonistic activity towards *S. aureus*. The highest activity towards *S. aureus* was obtained by the species *L. brevis* (FFC199 and SAU105 isolates). They showed halo of inhibitions greater than 3 mm (Table 2). The isolates *L. plantarum* CH3, *L. fermentum* CH58 and *L. brevis* SAU105 and FFC199 had activity towards *L. monocytogenes* 4446 (halo of inhibitions of 1-2 mm). The antagonistic activity found in this work is not associated to acids action since the assay was performed using the supernatant of the cells with pH adjusted to 7.0.

Table 2 Percentage ofco-aggregation of selected *Lactobacillus* isolates with *E. coli* JM109. The reference codes indicate the source of LAB: CH = cocoa fermentation, SAU = sausageand FFC = fermented food cauim

Strain	**% Co-aggregation with *E. coli* JM109 after 5 h**	**Antagonism activity**	
		***L. monocytogenes* 4446**	***S. aureus***
L. plantarum CH3	7.44 ± 0.22^{b}	+	-
L. plantarum CH41	30.67 ± 1.02^{e}	-	-
L. fermentum CH58	21.00 ± 1.11^{c}	+	+
L. brevis FFC199	1.09 ± 0.12^{a}	+	++
L. plantarum SAU96	21.08 ± 0.28^{c}	-	+
L. brevis SAU105	28.07 ± 0.43^{d}	+	+++

Presented values are means of triplicate determinations; ± indicates standard deviations from the mean. Mean values (± standard deviation) within the same column followed by different superscript letters differ significantly ($P < 0.05$) by Scott–Knott test. (+) = 1 - 2 mm; (++) = 3 – 4 mm; (++) = > 4 mm. No antagonism activity was observed towards others strains *E. coli* JM109, *S.* Typhimurium 224/87, *L. monocytogenes* EGDe and *L. monocytogenes* LO28

3.5. Adhesion to Caco-2 cells

The adhesion ability to Caco-2 cells was evaluated for the 6 selected LAB and the results are presented in Fig. 3. All 6 isolates showed a very low percentage of adhesion ability. However, the isolates *L. plantarum* SAU 96 and CH3 showed higher percentages (1.8 and 1.6 %of adhesion, respectively)of adhesion to Caco-2 cellscompared to the positive control *L. rhamnosus* GG (1.5 %). The isolates *L. plantarum* CH41, *L. brevis* FFC199 and *L. fermentum* CH58 showed moderated (1.1, 0.9 and 0.8 %, respectively) adhesion ability, while the *L. brevis* SAU105 (0.3 %) isolate showed very low percentage of adhesion to Caco-2 cells.

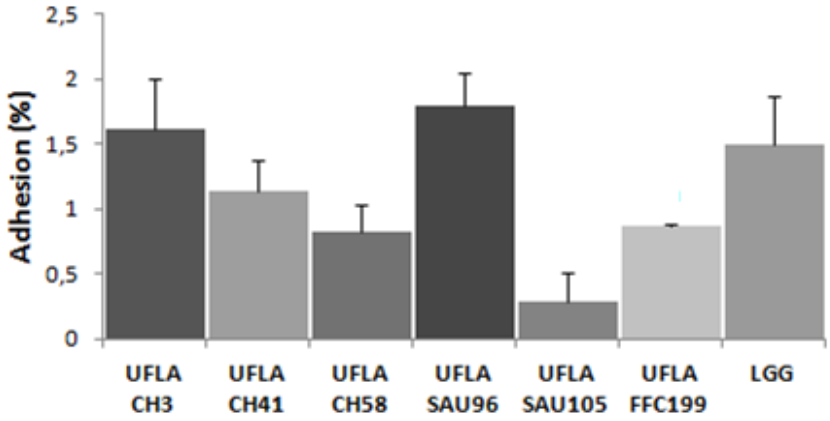

Figure 3 Percentage of adhesion of six selected LAB strains to Caco-2 cells. LGG was used as a reference strain. Bars indicate standard deviations from the media

3.6. TEER measurements

Changes in the TEER of polarized Caco-2 cells were used as an indicator of the effect of selected LAB strains on the intestinal epithelial barrier function (Fig. 4). All isolates maintained the TEER almost constant for the first 6 h, except for *L. plantarum* SAU96 isolate, which caused a slight increase in the first hour and a decrease in the TEER until 6 h. After 6 h, all of the tested isolates showed a tendency to increase the ratio of TEER. Compared to the control (Caco-2 cells without bacteria), the *L. plantarum* CH3 and CH41 and *L. brevis* FFC199 isolates induced a significant (p<0.05) increase in TEER after 24 h. The increase was similar to that caused by the reference probiotic strain *L. rhamnosus* GG.

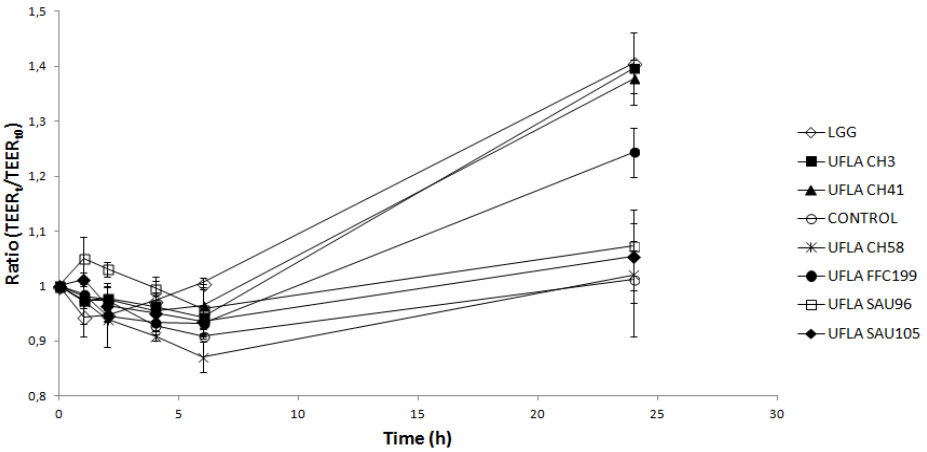

Figure 4 TEER of polarized Caco-2 monolayers exposed to 6 LAB strains at a concentration of 10^6 cfu mL^{-1}, or without bacteria (control). TEER is expressed as the ratio of TEER at time t in relation to the initial value (t0) for each strain. Bars indicate standard deviation from the mean. Lines followed by different letters differ significantly ($P < 0.05$) by Scott–Knott test

4. Discussion

Among 234 LAB previously isolated from 3 different products, 51 were selected based on their acid tolerance. Most of the isolates (43 out of 51) selected for further studies in this work originated from Brazilian cocoa fermentations. This is perhaps not surprising, as cocoa fermentations are characterized by low pH (approximately 2.0) (Nielsen et al., 2007; Pereira et al., 2012). The LAB isolated from cocoa are inherently able to survive the harsh fermentation conditions, and are, therefore, also likely to be able to survive the passage through the GIT.

Three different species were identified in the present study: *L. fermentum* (37 isolates), *L. plantarum* (10 isolates) and *L. brevis* (7 isolates). All the *L. fermentum* strains (34) and most of the *L. plantarum* isolates (9) identified in this work were isolated from cocoa fermentation. The results are in

agreement with observations of Nielsen et al. (2007) and Pereira et al. (2012), showing that *L. fermentum* and *L. plantarum* species are the dominant LAB in cocoa fermentations. Only one *L. plantarum* strain was isolated from sausage, while the *L. brevis* strains were isolated from both sausage (5 isolates) and cauim (2 isolates). These species have already been reported in these products (Almeida et al., 2007; Ammor & Mayo, 2007; Nobre et al., 2013; Ramos et al., 2010, 2011).

According to Fuller (1992), bile, even at low concentrations, can inhibit the *in vitro* growth of microorganisms. Gilliland et al. (1984) reported that 0.3 % is considered to be a critical concentration for screening for resistant strains. In the present study, all of the strains were able to grow in 0.3% (w/v) bile, although the time required to grow with and without bile was different between the isolates. Thus *L. plantarum* CH3, *L. plantarum* CH41 and *L. brevis* FFC199 had the highest bile tolerance, while isolates belonging to *L. fermentum* species (CH24, CH43, CH85 and CH88) and to *L. brevis* species (SAU62 and SAU254) had the lowest tolerance. Our results confirm previous reports showing that the tolerance is not necessarily related to the species of LAB, but may also be strain-specific (Maldonado et al., 2012).

Analyses of surface properties, including auto-aggregation and hydrophobicity showed that *L. plantarum* SAU96 and *L. fermentum* CH58 exhibited the highest auto-aggregation activity, while *L. plantarum* CH41 obtained the highest value for co-aggregation with *E. coli*. Regarding hydrophobicity, none of the isolates presented high capacity except for the *L. brevis* SAU105 isolate. Some authors have suggested that these surface properties correlate with their adhesive capacity (Kos et al., 2003; Kotzamanidis et al., 2010). However, here it seems that there is no correlation between surface properties and adhesion ability, since *L. plantarum* SAU96 had the highest rate

of adhesion to Caco-2 cells and at the same time it did not showed hydrophobicity capacity.

The *L. plantarum* CH3 and CH41 and *L. brevis* FFC199 isolates, as well as the *L. rhamnosus* GG positive control were able to increase the TEER ratio over 24 h of incubation. Jensen et al. (2012) demonstrated a tendency to increase the TEER polarized monolayers of Caco-2 cells from 6 to 24 h incubation for four *L. reuteri* isolates, whereas *L. rhamnosus* GG revealed a tendency to decrease the TEER over the same time period. Contrary to these observations, it was observed that several of the investigated LAB including *L. rhamnosus* GG caused a significant increase in the TEER of the polarized Caco-2 cell monolayer as compared to the control. Jensen et al. (2012) described that *L. plantarum* MF1298 decreased the TEER, whereas Klingberg et al. (2005b) showed that this strain caused the highest increase in the TEER of polarized Caco-2 monolayers. In concurrence with the observations by Klingberg et al. (2005b) and our study related the highest TEER over 24 h to *L. plantarum* isolates and this species was able to significantly increase the TEER. According to Anderson et al. (2010) induction of enhanced expression of genes involved in tight junction signaling is a possible mechanism by which *L. plantarum* MB452 improves intestinal barrier function.

Strains of *L. plantarum* have previously been proven to be able to survive gastric transit and colonize the intestinal tract of humans and other mammals (Georgieva et al., 2008; Mathara et al., 2008) and several strains have been used for the development of functional and therapeutic foods (Shah, 2007). Based on the results reported here *L. plantarum* CH3 and CH41from cocoa seemed to be suitable candidates to be used as probiotics, as they showed adhesion ability, increased the TEER of polarized Caco-2 monolayers significantly, showed moderate auto-aggregation, and a short time to adaptation to the bile. Similar findings for an increase in TEER of Caco-2 cells caused by a

L. plantarum isolate from silage were reported by Anderson et al. (2010), showing that different kinds of products can be a reservoir of potential probiotic strains.

The high hydrophobicity of *L. brevis* SAU105 did not correlate with the adhesion capability to Caco-2 cell. This isolate presented low rates of adhesion to Caco-2 cells, and low increase in the TEER of Caco-2 monolayers. Similar results were found by other authors (Mathara et al., 2008; Zago et al., 2011), confirming that hydrophobicity values do not correlate with adhesion properties. *L. brevis* FFC199 exhibited a short time of adaptation to the media containing bile, moderate auto-aggregation property, adhesion to Caco-2 cells, and causing a relatively high TEER of Caco-2 cells. Kos et al. (2003) found a relationship between auto-aggregationand adhesiveness of *L. acidophilus* M92, this relationship also seems to exist for the studied isolates. *L. brevis* has the GRAS status (EFSA, 2009) however, it is not typically used in probiotic products although Collins et al. (1998) have mentioned *L. brevis* in a list of strains used in probiotic products. *L. brevis* FFC199 may be considered a potential probiotic. Although *L. fermentum* is known probiotic species (Milkesaar & Zilmer, 2009), only one of our isolates (CH58) presented functional properties such as high auto-aggregation, co-aggregation with *E. coli* JM109, antagonistic activity towards *L. monocytogenes* 4446 and *S. aureus*, and adhesion capacity to Caco-2 cells. However, *L. fermentum* CH58 did not present significant ($P < 0.05$) increase in the TEER ratio.

This study revealed a considerable heterogeneity in probiotic properties among the different isolates of LAB. This fact was also observed by Kos et al. (2003). *L. brevis* FFC199 and *L. plantarum* CH3 and *L. plantarum* CH41 isolates displayed most of the probiotic characteristics and would be potential probiotic microorganisms.

In conclusion, potential probiotic isolates were identified and characterized from different fermented foods. Probiotic characteristics of the LAB isolates from cauim, cocoa fermentation and fresh sausage have not been studied before. It was clearly observed that microorganisms belonging to the same species may develop different mechanisms and present different characteristics. The results of this work provided a preliminary selection of some potential isolates, *L. brevis* FFC199 (from cauim) and *L. plantarum* CH3 and *L. plantarum* CH41 (from cocoa) to be used as probiotic, due to tolerance to low pH and bile salts, which are conditions imposed by the GIT environment. In addition, they were able to adhere to human intestinal epithelial cell line (Caco-2), and then increased the TEER of Caco-2 cell line. This study reveals the importance in searching by new potential strains to be used as probiotic in different products, adding nutritional values to these products. Further studies should be performed in order to understand the mechanism which is related to survival in GIT conditions.

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ARTIGO 2

REAL-TIME MONITORING OF THE EFFECT OF THE GASTROINTESTINAL ENVIRONMENT ON pH HOMEOSTASIS OF LACTIC ACID BACTERIA CELLS AS MEASURED BY FLUORESCENCE RATIO-IMAGING MICROSCOPY

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Abstract

In the present work a small scale *in vitro* model of the gastro-intestinal tract (GIT) was developed to obtain real-time observations of the pH homeostasis of single cells of two potential probiotic strains, *Lactobacillus plantarum* UFLACH3 and *L. brevis* UFLAFFC199 as a measure of their physiological state during GIT transit.Changes in the intracellular pH (pH_i) were determined using fluorescence ratio imaging microscopy (FRIM). A wide range of pH_i values (3.5-8.5) were measured by employing two pH sensitive fluorescent probes; 5(6)-carboxyfluorescein succinimidyl ester and 5(6)-carboxy-2′,7′-dichlorofluorescein diacetate succinimidyl ester. The pH_i measurements of the single cells revealed a highly heterogeneous population, with pH_i values ranging between 6.5–7.5 and 6.5-8.0 or higher during passage of saliva (pH 6.4) and intestinal juices (pH 6.4), respectively. The stressful conditions of the gastric juice (pH 3.5) produced two sub-populations. The pH_i of one sub-population ranged between 3.5-4.5, while that of the other ranged between 4.5-5.6. *L. brevis* UFLAFFC199 lowered its pH_i closer to the pH_{ex} when adding nutrients to the gastric juice, whereas this was not the case for *L. plantarum* UFLACH3. By use of FRIM and the combination of two derivatives of the same fluorophore, this study is the first to produce an *in vitro* GIT model enabling real time monitoring of pH homeostasis of single cells of *Lactobacillus* spp. and other Gram positive bacteria in response to the wide range of pH_{ex} of the GIT. The results are expected to be useful in evaluation of the probiotic potential of *Lactobacillus* spp.

Keywords: *Lactobacillus brevis, Lactobacillus plantarum, in vitro* gastro-intestinal model**,** pH_i, FRIM, CFDA-SE, CDCFDA-SE**.**

Resumo

No presente trabalho, um modelo em pequena escala do trato gastrintestinal (TGI) *in vitro* foi desenvolvido para observar, em tempo real, a homeostase do pH de uma única célula para duas estirpes potencialmente probióticas, *Lactobacillus plantarum* UFLACH3e *L. brevis* UFLAFFC199, como avaliação do estado fisiológico durante o trânsito TGI. Alterações do pH intracelular (pH_i) foram determinadas utilizando-se a técnica de *fluorescence ratio imaging microscopy* (FRIM). Uma ampla faixa de valores de pH_i (3,5-8,5) foi avaliada por meio do emprego de duas sondas fluorescentes sensíveis a pH, diacetato de carboxifluoresceína succinimidil éster e diacetato de carboxi-2′,7′-diclorofluoresceína succinimidil éster. Avaliações do pH_i das células individuais revelaram uma população altamente heterogenea, com pH_i variando entre 6,5-7,5 e 6,5-8,0 ou superior, durante a passagem da saliva (pH 6,4) e suco intestinal (pH 6,4), respectivamente. As condições estressante impostas pelo suco gástrico (pH 3,5) produziram duas subpopulações. O pH_i de uma subpopulação variou entre 3,5-4,5, enquanto da outra variou entre 4,5-5,6. *L. brevis* UFLAFFC199 reduziu seus valores de pH_i, para próximo do valor do PH_{ex}, quando uma solução nutriente foi adicionada ao suco gástrico, o que não ocorreu com as células de *L. plantarum* UFLACH3. Utilizando-se a técnica de FRIM e combinando-se dois derivados do mesmo fluoróforo, o presente estudo foi o primeiro a desenvolver um modelo *in vitro* do TGI, permitindo o monitoramento, em tempo real, da homeostase do pH de células individuais de *Lactobacillus* spp. e de outras bactérias gram-positivas, em resposta à ampla faixa $depH_{ex}$ do TGI. Os resultados poderão ser úteis na avaliação de *Lactobacillus* spp. potencialmente probiótico.

Palavras-chave: *Lactobacillus brevis*, *Lactobacillus plantarum*, modelo gastrointestinal *in vitro***,** pH_i, FRIM, CFDA-SE, CDCFDA-SE**.**

Introduction

The interest in functional foods containing probiotic microorganisms has increased significantly during the recent decade due to greater awareness of the benefits of probiotics for gut health, disease prevention and therapy (1, 2). Survival during passage of the upper part of the gastrointestinal tract (GIT) is considered important for the probiotics to exert their effects on the host (3). Several studies evaluating probiotic survival during GIT transit showed that the survival rate varies due to several factors, such as the probiotic strain and physiologic growth state (4, 5). Several *in vitro* models of the GIT have been developed for evaluating probiotic survival outcomes in this environment (6, 7, 8, 9, 10); however, a system for real-time monitoring of pH homeostasis of single cells as a measurement for their vitality has never been developed.

Information on the survival and/or vitality of microorganisms in different environments has traditionally been obtained at the population level, typically using plate count techniques and more recently e.g. PCR-DGGE (6, 7, 8, 9). This experimental approach, however, does not take into account the heterogeneity within an isogenic population of cells that arises from either mutations (genotypic) or progression through the cell cycle (phenotypic) under stressful conditions (11). Cell-to-cell variability may lead to the formation of sub-populations that may respond differently to stress conditions compared to the average cell population (12, 13). Therefore, there has been increased interest in methods that can establish the physiological status (i.e., viability/vitality) of individual cells within a population (14, 15, 16). One of these methods include Fluorescence ratio imaging microscopy (FRIM) (17). By FRIM it is possible to determine the intracellular pH (pH_i) in individual cells and thereby follow changes in pH homeostasis during changing extrinsic conditions (15, 18, 19). FRIM has previously been reported as a useful tool for assessment of the

physiological state (vitality) of individual cells (17), but most published studies are related to the food chain (13, 20) and no studies using FRIM for real-time monitoring of the response of single cells in the GIT environment have been published.

Several lactic acid bacteria (LAB) have been recognized as probiotics due to their health-promoting and nutritional properties (1). A correlation between a decrease in pH_i and a loss of vitality has been found for these bacteria (21, 22); and regulation of pH_i has been suggested as an important factor in the induction of tolerance to acid and other stress factors (23). Measurements of changes in the pH_i of individual bacterial cells based on staining with carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) have been used for different purposes in several studies (22, 23, 24, 25). However, the pH range that can be determined with carboxyfluorescein is only valid between pH 5 to 9 (17). Although this range is useful for many experiments, it is not sufficient for studies in a GIT environment, in which the pH easily may reach values way below 5. This problem can be overcome by using probes with a lower pK_a (15). The 5-(and -6)-carboxy-2′,7′-dichlorofluorescein diacetate succinimidyl ester (CDCFDA-SE) probe has successfully been employed as a pH_i indicator in bacteria under acid conditions (15).

The aim of this work was to develop a small-scale GIT system for real-time monitoring of the effect of gastrointestinal conditions on the pH homeostasis of individual bacterial cells via FRIM. The effect of simulated gastrointestinal conditions on pH_i was determined for the potential probiotic strains L. *plantarum* UFLA CH3 and *L. brevis* UFLA FFC199.

Materials and Methods

Strains and growth conditions. *Lactobacillus plantarum* UFLA CH3 and *Lactobacillus brevis* UFLA FFC199 (Ramos et al., unpublished data) were stored at -80 °C in Man Rogosa Sharpe broth (MRS, Merck, Darmstadt, Germany) with 20 % (v/v) glycerol. They were streaked onto MRS agar and incubated for 48 h at 37 °C. A loopful of cells was transferred to 10 mL MRS broth and incubated for 24 h (37 °C), after which 10 µl was transferred to 10 mL MRS broth and incubated for 18 h (37 °C).

Fluorescence labeling of *Lactobacillus* strains. Following propagation as described above, the *Lactobacillus* spp. strains were fluorescently labeled with a pH-sensitive probe, CFDA-SE (Molecular Probes Inc., Eugene, OR, USA), using a modified version of a previously published protocol (22).Briefly, cells (1 mL) were harvested by centrifugation at 10,000 x *g* for 2 min and 30 sec and re-suspended in sterile-filtered (0.22 µm; Millipore, Billerica, MA, USA) citric acid-phosphate-buffer (pH 5.8). Glucose and the probe CFDA-SE were added to a final concentration of 10 mM and 10 µM, respectively, and the cells were incubated at 37 °C for 30 min. Fluorescently labeled cells were harvested by centrifugation (10,000 x *g*, 2 min and 30 sec) and re-suspended in 1 mL of MRS broth.

The CDCFDA-SE (Molecular Probes Inc., Eugene, OR, USA) can be used to stain cells for pH_i measurements at low exterior pH values (15). Cells were prepared as described above for the CFDA-SE probe, except that the CDCFDA-SE probe was added to a final concentration of 39 µM.

Immobilization of cells for microscopy analysis. Cells were immobilized on the surface of a glass cover-slip, which had been cleaned with ethanol/HCL, thoroughly rinsed with sterile MilliQ-water and allowed to air dry. To facilitate the immobilization of the microorganisms, each cover-slip was

coated with 30 µL poly-L-lysine solution (0.1 % [w/v] aqueous solution, Sigma, St. Louis, USA) for 5 min, rinsed in sterile MilliQ-water and allowed to air dry. A CoverWell™ perfusion chamber gasket (2 chamber per slide, dimensions 19 x 6 x 0.5 mm; Molecular Probes) was placed on top of each cover-slip, and the chambers were then mounted as previously described (26) (Fig. 1). The stained cells (70 µL) were added to the chamber and centrifuged (2,000 x *g*, 30 sec) to facilitate cell attachment.

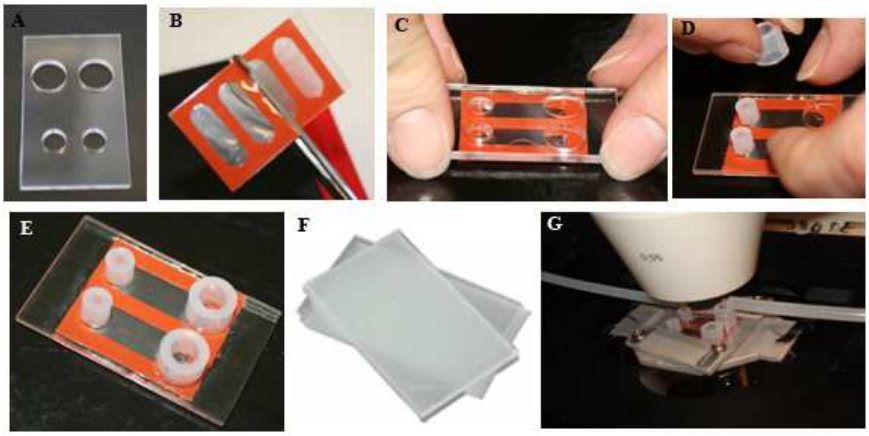

Figure 1 Illustrations of the assembly of the CoverWell perfusion chamber for flow experiments. (A) Acrylic glass with holes for inlet and outlet tubes. (B) The 4-chamber CoverWell perfusion was cut into two pieces, (C) on the plastic top of the chamber piecewas glued (using super-attack glue) on the Acrylic glass.The holes in the CoverWell perfusion chamber are in the middle of the inlet hole in the acrylic glass. (D) Inlet and outlet tubes were glued to the acrylic glass. (F) The cleaned and pre-treated (poly-L-lysine) cover-slip was glued to the acrylic glass. (G) The chamber was air driedfor 18 h and the CoverWell chamber was ready for use

Determination of the intracellular pH of immobilized *Lactobacillus* spp. cells. The pH_i of individual *L. plantarum*UFLA CH3 and *L. brevis* UFLA FFC199 cells stained with either CFDA-SE or CDCFDA-SE was measured by FRIM as previously described (19). The set-up consisted of an inverted epifluorescence microscope (Axiovert 135 TV; Zeiss, Birkerød, Denmark) equipped with a Zeiss Fluar 100 x objective (1.3 numerical aperture), a dichroic mirror (510 nm), and an emission band-pass filter (515 nm to 565 nm). Bacterial cells were excited at 488 nm and 435 nm with an exposure time of 3.000 ms by a monochromator that was equipped with a 75 W xenon lamp (Monochromator B; TILL Photonics GmbH, Planegg, Germany). Fluorescence emission was collected with a cool charge-coupled device camera (Coolsnap FX; Photometrics, Roper Scientific, Planegg, Germany), and images were analyzed with Metavue 7.1 software (Molecular Devices, Dowington, PA). Regions were defined for approximately 30 to 40 individual cells in each experiment using Metavue. A region near each cell but without a cell was subtracted for each cell as background. The ratio $R_{488/435}$ for each examined cell was obtained by dividing the fluorescence intensity at 488 nm (pH-sensitive wavelength) by the fluorescence intensity at 435 nm (pH-insensitive wavelength).

Standard curves for pHi measurements. Standard curves were constructed for each strain and probe as described previously (19). If a cell membrane is permeabilized, pH_iwill be equal to extracellular pH (pH_{ex}); thus, it was possible to establish the relationship between $R_{488/435}$and pH_i using the following approach: ethanol (70 %, vol/vol) was added to CFDA-SE- and CDCFDA-SE-stained *Lactobacillus* strains cells for 5 min to irreversibly permeabilize the cell membranes. Subsequently, the bacterial cells were harvested by centrifugation (10,000 × g, 2.5 min). The cells were resuspended in MRS broth whose pH was adjusted to a range of 3.5 to 8.0 (in 0.5 increments) after autoclaving. The curves were fitted to a 5^{th} order polynomium (Fig. 2).

Conversion was automatically performed with Microsoft Excel, and the ratio value for every cell at every time point was converted to pH_i.

Validation of probes. To validate and combine the results obtained using CFDA-SE and CDCFDA-SE probes, individual *L. plantarum* UFLA CH3 and *L. brevis* UFLA FFC199 cells were re-suspended in MRS broth whose pH was adjusted with either 1.0 M NaOH or concentrated (36%) HCl after autoclaving to a pH range of 3.5 to 8.0 (in 0.5 increments), and the cells were incubated for 30 min at 37 °C. The pH was measured by FRIM as described above using both probes. The assay was repeated twice using both probes

Effect of fluorescent labeling and nutrients on the survival of cells during passage of the GIT. Fluorescently labeled cells as well as non-labeled cells were inoculated into saliva (pH 6.4) at a level of 10^8 cells/mL and incubated for 5 min at 37°C. The cells were subsequently harvested by centrifugation (10,000 x *g*, 5 min) and re-suspended into gastric juice (pH 3.5, with or without nutrients) and incubated for 2.00 h before centrifugation (10,000 x *g*, 5 min) and re-suspending into the intestinal juice (pH 6.4) and incubating for another 2.00 h. Samples for determination of CFU/ml were taken immediately after inoculation/re-suspending (time 0) and immediately before centrifugation (time t). The percentage survival (% V_t) at time t was calculated using the following equation:

% V_t= CFU_tx 100 / CFU_0, where CFU_t, $CFU_0$is the CFU/mL obtained at time t and 0, respectively. Analyses of the variance and the Scott–Knott test were performed with SISVAR 5.1 software (27).

Simulated GIT. Gastrointestinal transit was simulated for the mouth, stomach, and small intestine. The experiments were performed at 37 °C. Transit times were chosen on a physiological basis as reported in the literature (28, 29) and were 5 min, 2.00 h, and 2.00 h for the mouth, stomach, and intestinal compartments, respectively. GIT juices were prepared as described by Oomen et

al. (30). The pH values of saliva, gastric juices and intestinal juices were 6.4, 3.5 and 6.4, respectively. The pH of the juices was selected based on a literature survey (31, 32, 33, 343, 35). Digestive juices were prepared the day before the digestion experiments. Soy peptone 3 g/L; glucose 0.4 g/L was prepared and added to the gastric juices to observe the effect of nutrient addition on pH homeostasis.

Real-time GIT System. A set-up similar to that employed by Guldfeldt and Arneborg et al. (36) was used with an inlet and outlet high-precision peristaltic pump to create a continuous flow in a perfusion chamberinoculated with stained cells prepared as described above. This system allowed for controlled flow and rapid switching between saliva and gastrointestinal juices. The juices were kept in a water bath at 37°C. Emission values obtained at excitation wavelengths of 435 nm and 488 nm were recorded as described above every 15 min, except for in saliva (5 min), to determine changes in the pH_i of single cells as a response to the changing extracellular environment. Measurement of the pH_i of the cells in gastric juice was obtained using the CDCFDA-SE probe, while the results for both saliva and intestinal juice were obtained using the CFDA-SE probe.

The survival of the cells in the GIT model was confirmed by staining the cells at the end of each experiment using 0.5 µM propidium iodide (PI; Molecular Probes Inc., Eugene, OR, USA), which was diluted in the intestinal solution, and observing them according to Smigic et al. (20).

Results

Correlation between pH_i and $R_{488nm/435nm}$ for CFDA-SE and CDCFDA-SE. Initially, calibration curves ($R_{488nm/435nm}$ versus pH_i) were constructed using ethanol-treated cells stained with the CFDA-SE and

CDCFDA-SE probes for both *L. plantarum* UFLA CH3 and *L. brevis* UFLA FFC199 cells (Fig. 2). The stained cells were re-suspended in MRS broth at different pH values. As shown in Fig. 1 the $R_{488nm/435nm}$ ratios were almost identical for the two *Lactobacillus* spp. strains.

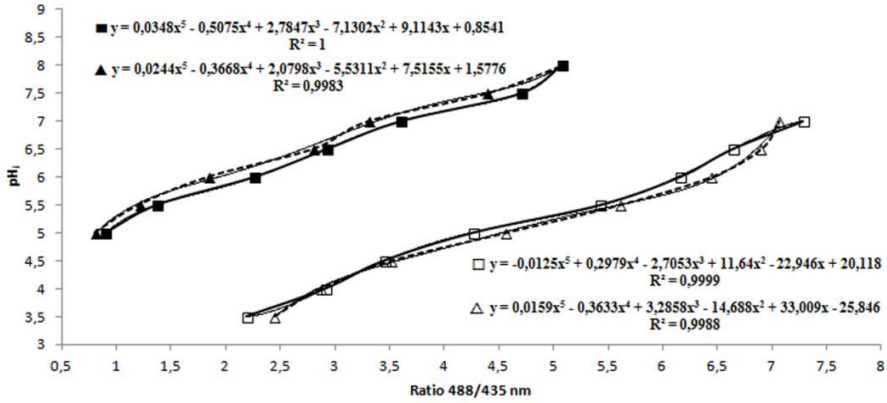

Figure 2 Correlation between the pH_i and $R_{488nm/435nm}$ for CFDA-SE- (black symbols) and CDCFDA-SE- (white symbols) -stained and -ethanol-permeabilized cells of *L. plantarum* UFLA CH3 (square) and *L. brevis* UFLA FFC199 (triangle)

Validation of probes. The results obtained for pH_i of viable cells of the *Lactobacillus* spp. strains in MRS broth stained with the two probes CFDA-SE and CDCFDA-SE were compared at pH_{ex} values of between 3.5 and 8.0 (0.5 pH increments). As shown in Fig. 3a (*L. brevis*) and 2b (*L. plantarum*) there was a good agreement in the measured pH_i values at pH_{ex} between 5.5 and 6.0 (both dyes can be used). Large standard deviations in pH_i were obtained with the CDCFDA-SE probe when the pH_{ex} was 6.5 and above. Thus the CDCFDA-SE probe gave more accurate pH_i measurements at pH_{ex} values below 6.0, whereas at pH_{ex} values of 5.5 or higher, the CFDA-SE probe gave more accurate pH_i results.

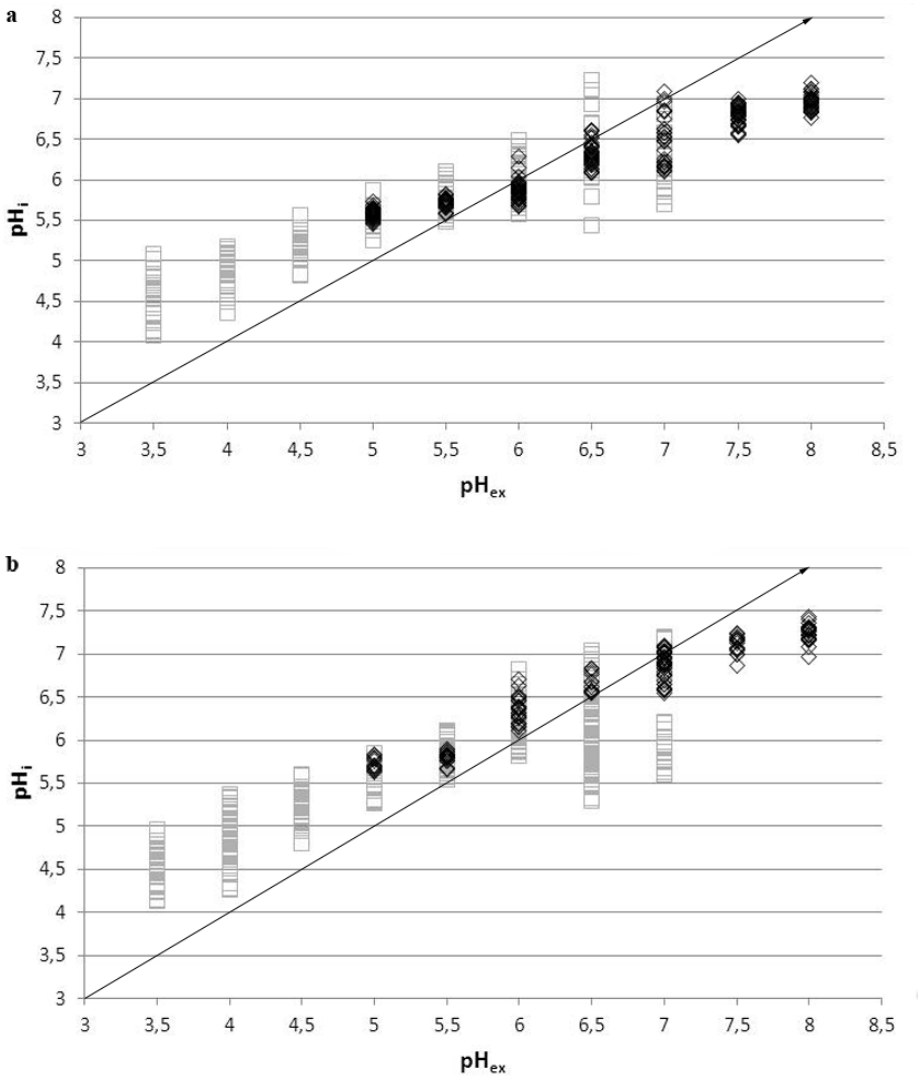

Figure 3 Measurements of pH_i in both *L. plantarum* UFLA CH3 and *L. brevis* UFLA FFC199 cells. The cells were stained with CFDA-SE (black diamond) and CDCFDA-SE (grey square) and subsequently re-suspended in MRS broth with the pH adjusted from 3.5 to 8.0. (a) *L. plantarum* UFLA CH3; (b) *L. brevis* UFLA FFC199. The arrows indicate $pH_{ex} = pH_i$

Effect of nutrients and florescent labeling on the survival of cells in the GIT model. Based on CFU/ml counts, there was no significant difference in percentage survival (approximately 90 % survival, $P<0.05$, Table 1) after exposure to saliva (5 min), gastric juice (120 min) and intestinal juice (120 min) regardless assay used (with or without nutrient addition). Further there was no significant differences ($P< 0.05$) observed in the percentage survival between fluorescently labeled cells and non-labeled cells during passage of the GIT (Table 1) indicating that staining does not influence the results for pH_i obtained with FRIM. However, there was significant difference ($P = 0.0007$) in survival percentage between the isolates *L. plantarum* UFLA CH3 and *L. brevis* UFLA FFC199 when exposed to gastric juice.

Table 1 Probability of the effects over the percentage of viability of stained and not stained cells in the GIT conditions with and without nutrient solution for *L. plantarum* UFLA CH3 and *L. brevis* UFLA FFC199

	P		
	Saliva	Gastric juice	Intestinal Juice
Isolate (I)	NS	0.0007	NS
Probe (Pr)	NS	NS	NS
Nutrient solution (N)	NS	NS	NS
I x Pr	NS	NS	NS
I xN	NS	NS	NS
Average of survival percentages	90.00	90.71(*L. plantarum* UFLA CH3) 93.33 (*L. brevis* UFLA FFC 199)	90.21

P means probability of the effects; I x Pr means interation between Isolate and Probe; I x N means interation between the Isolate and Nutrient solution; NS means no significant difference ($P < 0.05$) was observed by Scott–Knott test

Real-time GIT model. A real-time system to monitor the pH homeostasis of single cells using FRIM under conditions simulating passage of the GIT was developed and results obtained with the system are presented below. The experiments were made with or without addition of a nutrient solution to the gastric juice. Fig. 4 shows an example of differences in pH_i between single cells of *L. brevis* UFLA FFC199 exposed to saliva (Fig. 3a), gastric juice (Fig. 3b) and intestinal juice (Fig. 3c).

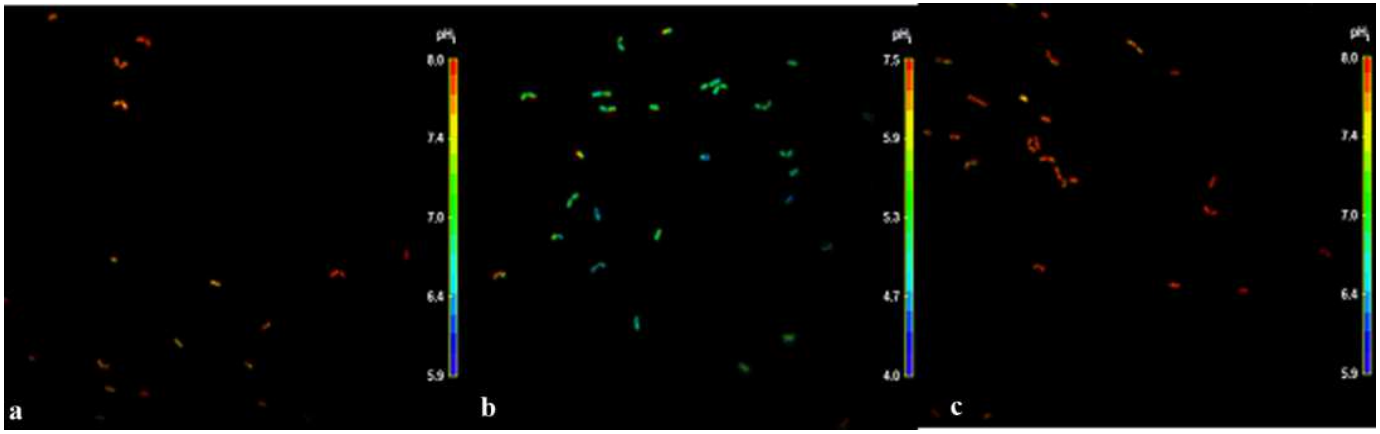

Figure 4 Photomicrografhs of fluorescence of *L. brevis* UFLA FFC199 cells analyzed by FRIM in the small-scale GIT model. (a) Cells exposed to saliva and stained with CFDA-SE. (b) Cells exposed to gastric juice and stained with CDCFDA-SE. (c) Cells exposed to intestinal juice and stained with CFDA-SE. The color bars correspond to the pH_i of the cells. Fig. 3a and c: pH from 5.9 to 8.0. Fig 3b: pH from 4.0 to 7.5

Fig. 4a and 4b shows the pH_i during passage of the GIT for *L. plantarum* UFLA CH3 and *L. brevis* UFLA FFC199, respectively, without the addition of a nutrient solution to the gastric juice. A rapid change in pH_i was observed for both strains studied as a function of the changes in the solutions and pH_{ex} (saliva pH 6.4, gastric juice pH 3.5 and bile/intestinal juice pH 6.4). The observed changes in pH_i differed among the individual cells, indicating that the responses of the bacterial cells were heterogeneous (Fig. 5). The pH_i observed for cells exposed to saliva (pH 6.4) was 6.5 to 7.0 for *L. plantarum* UFLA CH3 and 6.3 to 7.3 for *L. brevis* UFLA FFC199. Measurements of the pH_i by FRIM revealed the emergence of two sub-populations under the stressful conditions met in the gastric juice (pH 3.5). For one sub-population of cells the pH_i decreased to between 3.5 and 4.5, while for the other subpopulation the pH_i only decreased to between 4.5 and 5.6. For *L. brevis* UFLA FFC199 the sub-population with the lower pH_i regained a pH_i of 4.5 to 5.6 at 90 min of exposure to gastric juice (Fig 5b), whereas this was not the case for *L. plantarum* UFLA CH3 (Fig. 5a). However, the pH_i was for both lactobacilli regained after transfer to the intestinal juice (pH 6.4) where the pH_i increased to between 6.7 and 8.5 for *L. plantarum* UFLA CH3 and between 7.3 and 8.3 for *L. brevis* UFLA FFC199.

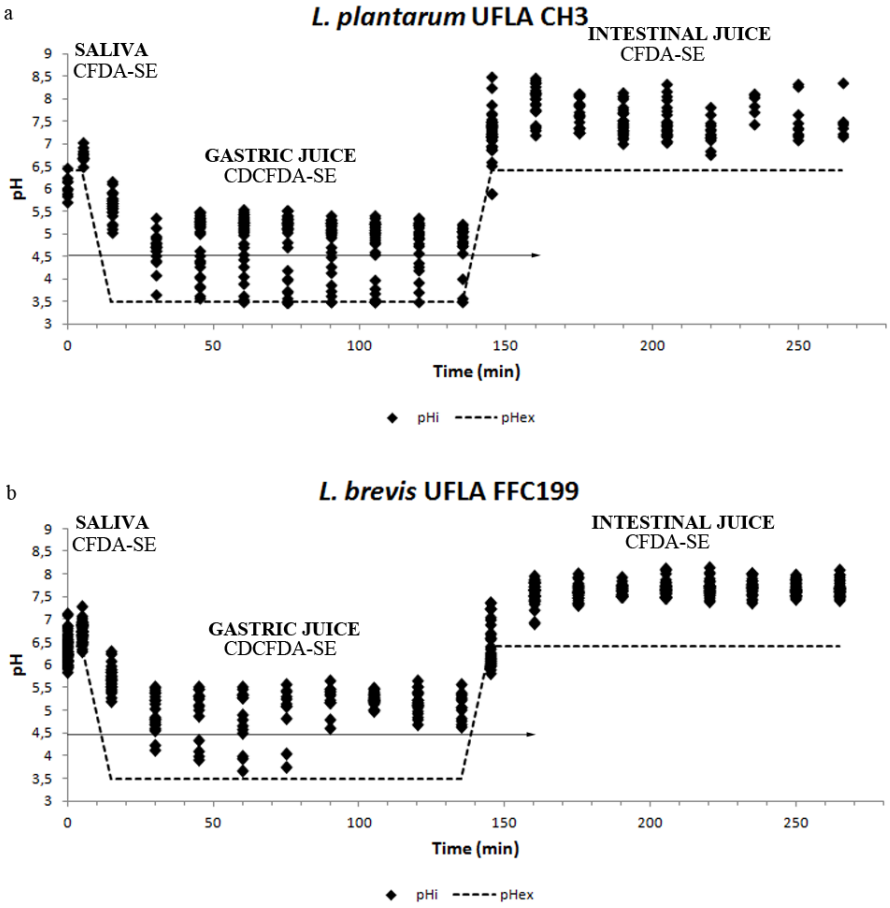

Figure 5 Changes in pH_i of *L. plantarum* UFLA CH3 (a) and *L. brevis* UFLA FFC199 (b) during passage of a small-scale GIT model (saliva (pH 6.4), gastric juice (pH 3.5) and intestinal juice (pH 6.4). The arrows indicate the emergence of two subpopulations occuring during passage of gastric juice

A nutrient solution was added to the small-scale GIT model to investigate whether it had any effect on the maintenance of the pH homeostasis of *L. plantarum* UFLA CH3 (Fig. 6a) and *L. brevis* UFLA FFC199 (Fig. 6b). For *L. plantarum* UFLA CH3 the pH_i values obtained with the addition of the

nutrient solution (Fig 6a) were similar to those obtained without nutrient solution (Fig 5a) throughout the GIT. For *L. brevis* UFLA FFC199 it was observed that the pH_i of the cells in gastric juice containing nutrients tended to approach the pH_{ex} (dotted line in the Fig. 6b) at 90 min. This pattern was not observed for cells in gastric juice without nutrient solution (Fig. 5b). In addition, when subsequently exposed to the intestinal juice, the cells were only capable of maintaining a ΔpH_i of 0.2 or higher in the assays with nutrients (Fig 6b), while it was around 0.9 or higher in the assays without nutrients (Fig. 5b). The Tab. 2 and Tab. 3 show the averages of $pH_i$and $\triangle pH_i$of the cells for both strains in the different solutions of the GIT model.

Table 2 Averages of pH_i of the lactobacilli cells in the different juices of the GIT model

Strain	**without nutrient solution**			**with nutrient solution**		
	Saliva	**Gastric**	**Intestinal**	**Saliva**	**Gastric**	**Intestinal**
L. plantarum UFLA CH3	6.4±0.4	4.9±0.6	7.5±0.4	6.7±0.2	4.9±0.6	7.6±0.4
L. brevis UFLA FFC199	6.5±0.3	5.2±0.4	7.5±0.4	6.3±0.3	5.0±2.6	7.2±0.5

Table 3 Averages of $\triangle pH_i$ (Average ± SD) of the lactobacilli cells in the different juices of the GIT model

	$\triangle pH_i$					
	without nutrient solution			**with nutrient solution**		
Strains	**saliva**	**Gastric**	**Intestinal**	**saliva**	**Gastric**	**Intestinal**
L. plantarum UFLA CH3	0.2±0.3	1.4±0.3	1.2±0.2	0.3±0.1	1.4±0.3	1.2±0.2
L. brevis UFLA FFC199	0.2±0.2	1.6±0.3	1.1±0.4	0.1±0.1	1.5±0.6	0.8±0.4

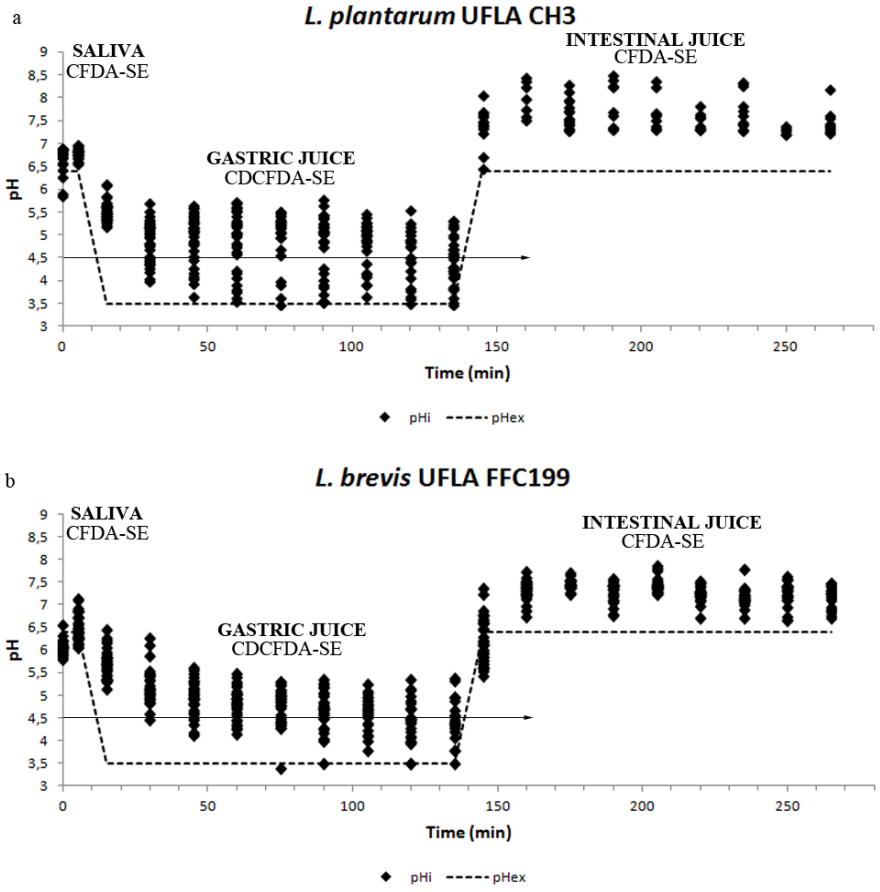

Figure 6 Changes in the pH_i of *L. plantarum* UFLA CH3 (a) and *L. brevis* UFLA FFC199 (b) during passage of a small-scale GIT system: saliva (pH 6.5), gastric juice (pH 3.5) and intestinal juice (pH 6.4) upon addition of a nutrient solution to the gastric juice. The arrows indicate the emergence of two subpopulations occuring during passage of gastric juice

For both strains most of the cells were able to maintain a pH gradient towards the surroundings throughout the experiment, indicating that during GIT conditions the strains remained viable and capable of actively pumping out protons from the cell. Viability was confirmed by staining the cells at the end of

the each experiment with propidium iodide (PI). An example is presented in Fig. 7, and shows that the majority of the cells remained viable at the end of the experiment because they were not stained by PI.

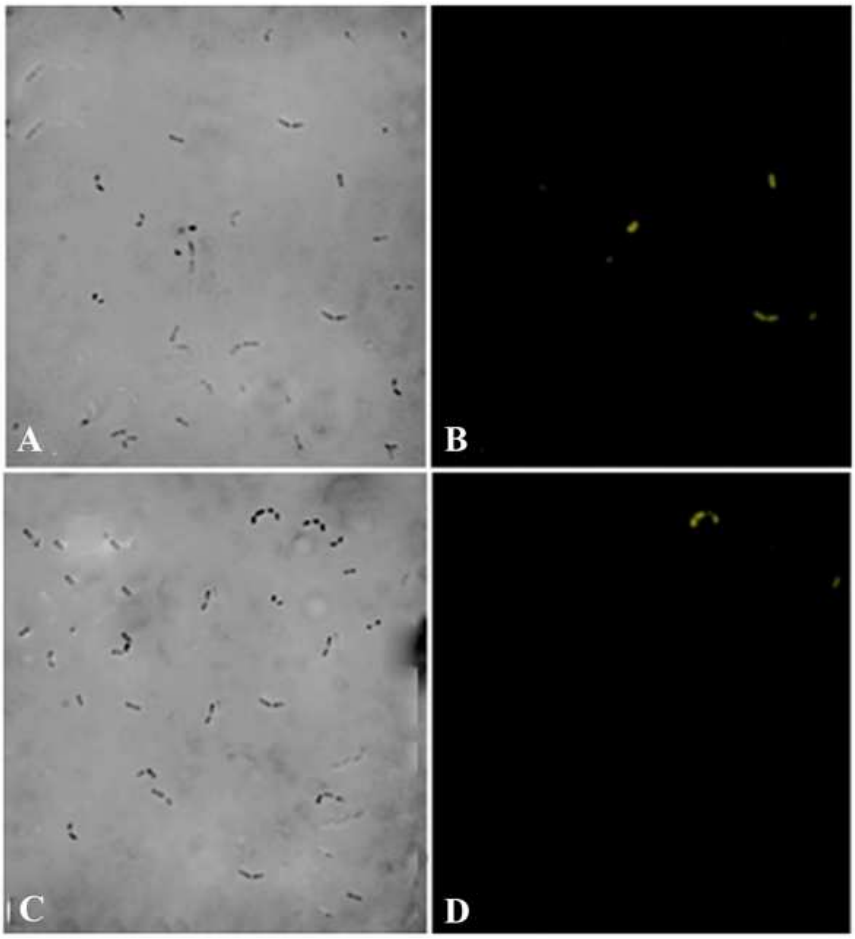

Figure 7 Light micrograph of *L. plantarum* UFLA CH3 (A) and *L. brevis* UFLA FFC199 cells (C). And epifluorescence microgrhaphof *L. plantarum* UFLA CH3 (B) and *L. brevis* UFLA FFC199 cells (D) (Axiovert 135 TV; Zeiss, Birkerød, Denmark) using a Zeiss Fluar 100 x objective. The cells are stained with propidium iodide (PI) at the end of the GITsystem experiment to observe their viability

Discussion

A small-scale GIT model combined with FRIM to monitor the effect of GIT passage on pH homeostasis of LAB at the single cell level was developed in the present study. Being the first study to use a combination of two pH sensitive probes, i.e. CFDA-SE and CDCFDA-SE, to determine the pH_i of single cells over a wide range of pH_{ex}, several measures were taken to validate their combined use. Apart from the difference in pK_a values of CFDA-SE and CDCFDA-SE, the presence of two chlorine atoms in the CDCFDA-SE probe ($C_{29}H_{17}Cl_2NO_{11}$) could influence the relative emission pattern. However, this did not pose a problem, since the ratios could be reproducibly correlated to unique pH values for both probes (Fig. 2). Our results confirm those of previous works, where both probes separately have been used successfully to measure the pH_i of single cells (15, 19, 22, 23, 24, 25).One purpose of this study was as indicated above, to investigate if the two probes CFDA-SE and CDCFDA-SE could be used in combination to enable valid pH_i measurements over a broad pH_{ex} range (4.0 – 8.0). Several authors (19, 22, 23, 25, 36) reported a pH_i value of 5.5 as the lower limit of sensitivity of CFDA-SE, which was also observed in the calibration curves for this probe in this study. Shabala et al. (15) demonstrated that CDCFDA-SE was suitable for obtaining measurements of pH_i as low as pH_i 4.0 in individual bacterial cells (pH 3.5 in the present study). Furthermore, the measurements of pH_i in both *L. plantarum* UFLA CH3 (Fig. 3a) and *L. brevis* UFLA FFC199 (Fig. 3b) cells using CDCFDA-SE and CFDA-SE overlapped between pH 5.5 and 6.0. Altogether, the results obtained suggest that the probes can be used in combination, enabling pH_i measurements at a pH_{ex} of between 3.5 and 8.0.

In the GIT model, a ΔpH with higher pH_i than pH_{ex} was observed for both *Lactobacillus* spp. in all juices (pH 3.5, pH 6.4). In comparison when the

pH_i of the two *Lactobacillus* spp. strains was measured in MRS with the pH adjusted to between 3.5 and 8.0 (0.5 increments), at pH_{ex} of 6.5 and above (*L. plantarum* UFLA CH3) and 7.0 and above (*L. brevis* UFLA FFC199), the cells maintained a pH_i which was lower than the pH_{ex} ($\Delta pH > 0.3$), whereas at acidic pH_{ex} of 3.5-5.5 the opposite was observed ($\Delta pH > 0.1$). Generally, the difference between the intracellular and extra-cellular pH increased when approaching the pH extremes of 3.5 and 8.0. Similar results were obtained by Zhang et al. (38) for *Lactobacillus casei*, showing an increasing ΔpH of 0.06, 0.25 and 0.38 with decreasing pH_{ex} values of 6.5, 5.0 and 3.5, respectively. Further, the results obtained in our study for the lower pH_i relative to the pH_{ex} are in agreement with pH_i measurements obtained for different *Lactobacillus* spp. strains at alkaline pH_{ex} of up to 9.0 by Sawatari and Yokota (24). Sawatari and Yokota (24) suggested that, the reversed ΔpH formation in the *Lactobacillus* spp. strains at alkaline pH_{ex} could be due to physicochemical forces (the Donnan potential) as well as the use of energy-dependent $Na^+(K^+)/H^+$ anti-porters. It probably presents different activities depending on the bacterial species and acidic/alkalinizing agent used to pH adjustments. Shabala et al. (15) also found different effect on the pH_i homeostasis depending on the acidic agent used. Although, in our study, only HCl and NaOH were the acidic/alkalinizing agents employed for pH adjustments, MRS is a rich media containing acetic acid. The acetic acid will be un-dissociated at low pH, and glucose present in the media can be fermented to lactic acid, affecting the *Lactobacillus* spp. cells(39). It could explain why the cells in the MRS broth and GIT model possessed different pH homeostasis, since the cells were propagated in the same way.

In the GIT model we were able to perform real time measurements of changes in the pH_i of the *Lactobacillus* spp. strains in response to the changes in the pH_{ex}. In the GIT model, shifting from saliva (pH 6.4) to gastric juice (pH 3.5) induced a rapid decrease in pH_i from 6.5-7.0 (*L. plantarum* UFLA CH3)

and 6.3-7.3 (*L. brevis* UFLA FFC199) to 3.5-5.5 in both strains. The almost immediate decrease in pH_i in the *Lactobacillus* spp. strains as a response to a sudden decrease in pH_{ex} has previously been observed by others, e.g. for *L. delbrueckii* subsp. *delbrueckii* (15, 23). Usually, in growing cultures of fermentative lactic acid bacteria (LAB) the pH_i will decrease as a function of pH_{ex}, and average ΔpH of 0.5 to 1.0 units have been reported (40,41, 42). Shabala et al. (15) however, observed no more than a ΔpH of 0.1 upon acid treatment for an acid tolerant *L. delbrueckii* subsp. *delbrueckii* strain, when the pH_{ex} was instantly lowered from 6.0 to 4.0. For the less acid tolerant *Listeria innocua* a relatively constant pH_i level with ΔpH 1.5 to 1.8 have been reported (15). In comparison, in the present study the ΔpH of the two *Lactobacillus* spp. strains ranged between 0.1- 2.0 in the gastric juice. Exposure to low pH negatively affects the proton motive force (PMF) across the cell membrane as a result of the accumulation of protons inside the cell (39). In response to acid stress, lactic acid bacteria employ numerous mechanisms to survive (39). Zhang et al. (38) found that acid tolerant *L. casei* were able to maintain higher amounts of ammonium at pH 3.5, which contributed to neutralize protons and the concomitant maintenance of the pH_i homeostasis. This and other mechanisms such as e.g. the proton translocating H^+-ATPase enzyme complex and membrane permeability (37) could be differently active in the *Lactobacillus* spp. individual cells, resulting in the observed emerging sub-populations under exposure to the low pH values in the gastric juice. Our results are in agreement with previous studies, e.g. by Hornbaek et al. (13) who observed the emergence of two sub-populations of *Listeria monocytogenes* at sub-inhibitory concentrations of bacteriocins, where one showed a dissipated ΔpH and the other maintained a constant ΔpH. Furthermore, adaptative evolution of microorganisms under stress conditions enhance the stress tolerance of the cell as observed by Zhang et al.

(38), where acid adapted cells (64 h at pH 4.3) of *L. casei* showed a 318-fold higher survival rate than that their parental cells in acid stress (3 h at pH 3.3). Several studies have examined the response of LAB to acidic stress, including the mechanisms underlying tolerance to organic acid. In comparison, few studies have examined the stress response of LAB to bile and how it affects the pH homeostasis of single cells (39, 43). Intracellular dissociation of bile salts may impose a low pH stress on cells and the movement of ions may have osmotic effects (44). As bile can chelate calcium and iron (45, 46) the presence of bile inside a cell may result in low intracellular calcium and iron concentrations. In the present study, when the cells were shifted from the gastric juice (pH 3.5) to the intestinal juice containing bile (pH 6.4), a ΔpH ranging from 0.3 to 2.0 (*L. plantarum* UFLA CH3) and 0.9 to 1.9 (*L. brevis* UFLA FFC199) was observed. In comparison, in the study by Sawatari and Yokota (24) a ΔpHof approximately 0.5 at pH_{ex} of 6.5 (without bile) for different *Lactobacillus* spp. strains (*L. acidophilus* JCM 1132, *L. mali* JCM116T, *L. plantarum* NRIC 0380, *L. curvatus* AHU GD12 and *L. paracasei* subsp. *tolerans* NRIC 1940) was observed.

It is known that beverages such as yoghurts could have a protective function enhancing survival of bacteria in acid environments (47, 48). However, the effect of nutrients on pH_i of bacterial cells in the GIT remains largely unknown (49). Although there was found no differences in the pH_i for *L. plantarum* UFLA CH3 cells with and without nutritious solution (glucose and peptone), addition of the nutrient solution to the gastric juice resulted in a general decrease (at 90 min onwards) of the ΔpH of the *L. brevis* UFLA FFC199 cells as compared to without nutrient solution. The glucose present in nutrient solution may be fermented to lactic acid by *Lactobacillus* spp. cells and due to the low pH in gastric juice, it will be difficult for the cell to export the lactic acid which will then lead to a lower pH_i (low pK_a value of lactic acid of 3.85). This could stress the *L. brevis* UFLA FFC199 cells, which is then reflected later,

when they are exposed to the intestinal juices. Further, it has been shown by Shabala et al. (15) that acidification with lactic acid affects the pH_i homeostasis more than HCl. In contrast, glucose may provide ATP by glycolysis, which could be used by energy dependent proton pumps to expel ions H+, increasing the pH_i of the cells. In the intestinal juice, the *L. brevis* UFLA FFC199 cells exposed to gastric juice without nutrient solution exhibited a higher ΔpH (0.9 or higher) than those containing nutrient solution (ΔpH 0.2 or higher). In other words, the *L. brevis* UFLA FFC199 cells had a low pH_i in the intestinal juice in the presence of nutrients and their pH_i was thereby closer to the pH_{ex}. Although, difference in survival percentage was not observed by CFU/mL counts regarding nutrient solution, there was difference between the isolates when exposed to gastric juice. Probably these species have different responses to survive in stress conditions caused by gastric juice.

For both *L. plantarum* UFLA CH3 and *L. brevis* UFLA FFC199 heterogeneity in pH homeostasis was observed among the cells upon passing through the GIT model. The heterogenic response found in the present work is likely related to the presence of naturally resistant cells in a better physiological condition (38). Phenotypic heterogeneity constitutes the non-genetic variation existing among individual cells within an isogenic population and is related to different levels of basal gene expression rather than different gene possession (11). According to Balázsi et al. (49) cells with identical genomes exposed to the same environment do not necessarily have identical phenotypes. These authors report that when cells assume different, functionally important and heritable fates without an associated genetic or environmental difference, cellulardecision making occurs (49). Waddington's epigenetic landscape (balls rolling down a slanted landscape with bifurcating valleys) has been widely and repeatedly used for several decades as a pictorial illustration of differentiation in multicellular development (50). Balázsi et al. (49) purposes that intrinsic gene expression

noise is a component missing from Waddington's epigenetic landscape. This is the reason in which identical cells released from the same location (Waddington's epigenetic landscape) will fell the perturbing effects of onnipresent random fluctuations at every point on their way. Random noise will shake them apart, modifying their trajectories and forcing them to cross barriers, diffuse along plateus, and find new local minima. Thus, intrinsic noise enables the phenotypic disversification of completely identical cells exposed to the same environment and further facilitates cellular decision making for cells already slightly different when release onto Waddington's landscape (49). In our study the pH_i homeostasis may be considerate the noise as well as observed in other studies when cells were exposed to stressful conditions such as heat, bacteriocins, RNA content, and levels of carotenoid pigmentation (13, 18, 51, 52). Therefore the variation observed in pH_i among the individual cells passing through the GIT model is not surprising.

In conclusion, a novel GIT model was developed and successfully applied in this work. The results obtained for both *L. plantarum* UFLA CH3 and *L. brevis* UFLA FFC199 showed that individual cells may maintain their pH_i differently. The juices in the GIT model affected the pH_i homeostasis of the *Lactobacillus* spp. cells, providing two sub-populations during exposure to gastric juice. The potential probiotics *L. plantarum* UFLA CH3 and *L. brevis* UFLA FFC199 showed to be robust strains, keeping their viability/vitality in the wide range of pH_{ex} imposed by the GIT. The nutritious solution employed in this work affected the pH_i maintenance of *L. brevis* UFLA FFC199 differently than *L. plantarum* UFLA CH3. This is the first study in which a small-scale GIT model has been used to monitor the physiological status of individual cell through pH_i measurements in real time. In addition, this is the first work in which both CFDA-SE and CDCFDA-SE probes were combined to study the pH_i in a wide range of pH_{ex} values (3.5 to 6.4). Therefore, this work provided results

that established a valid system that can be applied to several types of microorganisms to assess their behavior via pH_i in a GIT model. Also the model can be used to evaluate the probiotic potential of lactic acid bacteria, since their survival in GIT conditions is an important criterion to considerate a strain as probiotic.

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Considerações finais

Probióticos são microrganismos amplamente utilizados como suplementos alimentares ou adicionados a produtos alimentícios, no intuito de gerar benefícios à saúde. Alimentos naturalmente fermentados são considerados ricas fontes de microrganismos, cujas atividades funcionais ainda são pouco conhecidas.

As estirpes microbianas diferem na sua habilidade em superar as condições adversas, tais como baixo pH e presença de bile, impostas pelo TGI. Também diferem em suas propriedades funcionais, tais como adesão às células intestinais, aumento da resistência elétrica da barreira transepitelial e combate à microrganismos patogênicos. Por estas razões, o estudo das características probióticas dos microrganismos presentes em produtos naturalmente fermentados torna-se tão importante. Adicionalmente, avaliações da homeostase do pH_i é uma maneira eficiente de avaliar as condições fisiológicas celular, sendo possível acompanhar este comportamento em uma única célula por meio do método de FRIM.

Microrganismos com características potencialmente probióticas foram observados no presente estudo. Processos fermentativos, tais como fermentação de cacau e cauim, podem favorecer a 'seleção natural' de microrganismos mais resistentes. Durante a fermentação, podem ocorrer condições desfavoráveis para certos microrganismos, como, por exemplo, um ambiente competitivo, baixos pH e elevadas temperaturas, que podem favorecer a sobrevivência desses microrganismos às severas condições impostas pelo sistema gastrintestinal do hospedeiro.

Os resultados apresentados neste trabalho permitiram estabelecer um sistema válido para estudos do comportamento (homeostase do pH_i) de vários tipos de microrganismos nas condições do TGI. O modelo *in vitro* do TGI

utilizando FRIM possibilitou análises das condições fisiológicas de células únicas em tempo real e, assim, a observação da formação de subpopulações, quando submetidas às condições estressantes impostas pelo suco gástrico.