HUGO CALIXTO FONSECA

FRUIT JUICES AS CARRIER MATRICES OF POTENTIALLY PROBIOTIC BACTERIA

LAVRAS-MG
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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Ciência dos Alimentos, área de concentração em Microbiologia de Alimentos e Processos Fermentativos, para a obtenção do título de Doutor.

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APROVADA em 06 de abril de 2021.
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A minha família, em especial minha mãe Yara, minha filha Lavínia, minha irmã Silvana e minha namorada Bárbara, mulheres que sempre estão do meu lado, que com seus sentimentos de amor me encorajou a persistir na conquista dos objetivos.

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Dedico

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"*Não há transição que não implique um ponto de partida, um processo e um ponto de chegada. Todo amanhã se cria num ontem, através de um hoje. De modo que o nosso futuro baseia-se no passado e se corporifica no presente. Temos de saber o que fomos e o que somos, para sabermos o que seremos." (Paulo Freire)*

RESUMO

A fermentação por bactérias ácido-láticas probióticas é reconhecida por seus potenciais benefícios à saúde. A maioria dos produtos probióticos no mercado são derivados do leite, porém, há uma demanda crescente por alimentos probióticos à base de vegetais. Entretanto, como diversos parâmetros influenciam no processo de fermentação, as escolhas corretas do tipo de substrato e da cepa são primordiais no sucesso de obtenção de um produto final com características desejáveis. Com isso, o primeiro objetivo deste estudo foi avaliar as propriedades probióticas de 19 bactérias ácido-lácticas pertencentes à Coleção de Cultura da Microbiologia Agrícola (CCMA), usando ensaios de resistência, segurança e funcionalidade, definindo a cepa com maior potencial probiótico. *Lactiplantibacillus plantarum* CCMA 0743 teve alta taxa de sobrevivência em sucos gástrico e intestinal simulados e exibiu maior hidrofobicidade (96,06%). Nos ensaios simulados de competição e exclusão, a cepa CCMA 0743 foi capaz de reduzir a adesão de *Salmonella* em ambos os modelos celulares (Caco-2 e HT-29) e a adesão de *Escherichia coli* a células HT-29 no ensaio de competição. Na segunda parte, foram avaliadas as modificações fitoquímicas dos sucos de acerola, coquinho azedo e maracujá, fermentados por culturas simples e mista de *L. plantarum* CCMA 0743 e *Lacticaseibacillus paracasei* LBC-81 a 37 °C/24 h para definição da melhor matriz fermentativa. Após 18 h de fermentação, o suco de maracujá apresentou maior viabilidade da cultura de *L. plantarum* CCMA 0743 simples ou mista, acima de 9,00 Log UFC/mL e pH entre 4,07-4,10. O consumo de açúcares e a produção de ácidos orgânicos foram influenciados pelo substrato e pela cultura utilizada. O processo fermentativo reduziu a atividade antioxidante e o teor de carotenoides. No entanto, a cultura isolada de *L. plantarum* CCMA 0743 aumentou o teor de flavonoides amarelos no suco de maracujá. Por fim, o estudo investigou o efeito das cepas *Lactobacillus* nos perfis de compostos voláteis e sensorial do suco de maracujá. Além disso, a viabilidade das cepas foi avaliada em condições gastrointestinais simuladas e armazenamento refrigerado do produto. *L. plantarum* CCMA 0743 apresentou alta viabilidade (6,18 Log UFC/mL) na etapa final da digestão simulada do suco de maracujá. Ambos os sucos, de fermentação simples ou mista, mantiveram contagens adequadas (> 8,0 Log UFC/mL) durante o armazenamento e a cor amarela foi estável por 28 dias. O perfil sensorial do suco de maracujá foi modificado pelas fermentações simples e mista. De acordo com os resultados obtidos, *L. plantarum* CCMA 0743 mostrou-se excelente candidata para uso como probiótico, apresentou alta viabilidade celular e aumento de compostos bioativos em suco de maracujá. De maneira geral, o suco de maracujá se mostrou uma matriz alimentar adequada para fermentação das linhagens estudadas e produção de uma bebida com características físico-químicas, microbiológicas e sensoriais desejáveis.

Palavras-chave: Alimentos Funcionais. Conservação. Microbiologia. Perfil Rápido. Triagem.

ABSTRACT

Fermentation by probiotic lactic acid bacteria is recognized for its potential health benefits. Most probiotic products on the market are derived from milk, but there is a growing demand for plant-based probiotic foods. However, as several parameters influence the fermentation process, the correct choices of the type of substrate and the strain are paramount in the success of obtaining a final product with desirable characteristics. Thus, the first objective of this study was to evaluate the probiotic properties of 19 lactic acid bacteria belonging to the Agricultural Microbiology Culture Collection (CCMA) using resistance, safety and functionality tests, defining the strain with greater probiotic potential. *Lactiplantibacillus plantarum* CCMA 0743 had a high survival rate in simulated gastric and intestinal juices and exhibited greater hydrophobicity (96.06%). In the simulated competition and exclusion assays, strain CCMA 0743 was able to reduce the adhesion of *Salmonella* in both cell models (Caco-2 and HT-29) and the adhesion of *Escherichia coli* to HT-29 cells in the competition assay. In the second part, the phytochemical changes of the juices of acerola, sour coconut and passion fruit were evaluated by simple and mixed cultures of *L. plantarum* CCMA 0743 and *Lacticaseibacillus paracasei* LBC-81 at 37 °C/24 h to define the best fermentative matrix. After 18 h of fermentation, the passion fruit juice showed greater viability of the culture of *L. plantarum* CCMA 0743 single or mixed, above 9.00 Log CFU/mL and pH between 4.07-4.10. Sugar's consumption and organic acid production were influenced by the substrate and the culture used. The fermentative process reduced the antioxidant activity and the carotenoid content. However, the isolated culture of *L. plantarum* CCMA 0743 increased the content of yellow flavonoids in the passion fruit juice. Finally, the study investigated the effect of *Lactobacillus* strains on profiles of volatile compounds and sensory of passion fruit juice. Finally, the viability of the strains was evaluated under simulated gastrointestinal conditions and refrigerated storage of the product. *L. plantarum* CCMA 0743 showed high viability (6.18 Log CFU/mL) in the final stage of the simulated digestion of passion fruit juice. Both juices, single or mixed fermentation, maintained adequate counts (> 8.0 Log CFU/mL) during storage and the yellow color was stable for 28 days. The sensory profile of passion fruit juice was modified by simple and mixed fermentations. According to the results obtained, *L. plantarum* CCMA 0743 proved to be an excellent candidate for use as a probiotic, showed high cell viability and increased bioactive compounds in passion fruit juice. In general, passion fruit juice proved to be an adequate food matrix for fermentation of the studied strains and production of a drink with desirable physicochemical, microbiological, and sensory characteristics.

Keywords: Functional foods. Conservation. Microbiology. Flash Profile. Screening.

SUMÁRIO

PRIMEIRA PARTE – Introdução Geral

1. INTRODUÇÃO

Os produtos lácteos são tradicionalmente considerados os melhores transportadores de bactérias probióticas. No entanto, nos últimos anos, houve um aumento na demanda dos consumidores por alimentos probióticos não lácteos e, consequentemente, o desenvolvimento de produtos à base de vegetais, como os sucos de frutas, tem se tornado promissor (ASPRI; PAPADEMAS; TSALTAS, 2020).

Neste contexto, as frutas encontradas no Cerrado, nativas ou cultivadas, tornam-se alternativa na elaboração de sucos carreadores de probióticos. Dentre as diversas opções disponíveis, o maracujá, a acerola e o coquinho azedo apresentam relevantes valores nutricionais e funcionais devido a seus respectivos teores de compostos bioativos (DA SILVA *et al*., 2014; MAGALHAES *et al*., 2017; RUFINO *et al*., 2010). Além dos diversos nutrientes que podem ser fornecidos na dieta, os sucos de frutas possuem a capacidade de redução de peso pela modulação da microbiota intestinal, aumento da vasodilatação e diminuição da oxidação lipídica (HENNING *et al*., 2017).

Com isso, recentemente, diversos estudos sobre a fermentação de sucos de frutas por bactérias probióticas têm sido reportados na literatura (BONTSIDIS *et al*., 2021; DE OLIVEIRA *et al*., 2020; WU *et al*., 2021). Como resultados obtidos, as mudanças no perfil sensorial do produto (WU *et al*., 2021) e o aumento da concentração de compostos bioativos (KWAW *et al*., 2018; MOREIRA *et al*., 2017) merecem ser mencionados. Adicionalmente, outros trabalhos concluíram que pode existir a formação ou degradação de compostos após a fermentação, e que essas mudanças são dependentes da cepa utilizada e do seu metabolismo durante o armazenamento (ALVES FILHO *et al*., 2017; HASHEMI *et al*., 2017; NEMATOLLAHI *et al*., 2016), resultando, assim, em produtos com características físico-químicas e sensoriais diferenciadas.

Apesar dos sucos de frutas representarem interessantes matrizes para a adição de probióticos, sempre há a necessidade de avaliar a sobrevivência da cultura probiótica e seu impacto nas características de qualidade do produto (PIMENTEL *et al*., 2021). Devido a este impacto ser dependente da cepa e do substrato utilizados, estudos com matrizes à base de vegetais têm sido realizados, tanto com culturas probióticas comerciais, quanto com cepas selvagens potencialmente probióticas (PIMENTEL *et al*., 2021).

Especificamente quando se utiliza culturas não comerciais, suas propriedades probióticas podem ser avaliadas, uma vez que estas características são específicas de cada cepa.

Para isso, alguns testes *in vitro*, como a capacidade de tolerância às condições adversas do trato gastrointestinal, capacidade de adesão às células epiteliais, atividade antagonista contra patógenos, além dos critérios de segurança para consumo humano e propriedades tecnológicas, são relevantes na caracterização e seleção de novos probióticos em potencial (SHOKRYAZDAN *et al*., 2017). Finalmente, apesar dos desafios tecnológicos, o mercado de produtos probióticos não lácteos possui considerável capacidade de expansão, uma vez que estudos recentes têm mostrado o sucesso da adaptação de diferentes cepas em matrizes alternativas (ASPRI; PAPADEMAS; TSALTAS, 2020).

2. REFERENCIAL TEÓRICO

2.1. Probióticos: definições e atualizações

O mercado de alimentos contendo probióticos possui uma tendência de crescimento, devido ao aumento do interesse dos consumidores em hábitos alimentares mais saudáveis (BINDA *et al*., 2020). Em 2013, a Associação Científica Internacional de Probióticos e Prebióticos (ISAPP, por suas siglas em inglês) convocou um Painel de Especialistas para revisar o termo probiótico e a literatura que o cerca. O resultado foi uma publicação, reiterando a definição com pequenas alterações gramaticais: "Microrganismos vivos que, quando administrados em quantidades adequadas, conferem um benefício à saúde do hospedeiro" (HILL *et al*., 2014). Esta é a definição científica amplamente aceita em todo o mundo.

Os microrganismos pertencentes ao grupo de bactérias ácido-láticas (BAL) têm recebido valiosa atenção como probióticos nos últimos anos. Cepas BAL tradicionais têm uma longa história de uso seguro e amplo na indústria de alimentos, além de diversos efeitos benéficos reportados (KHANEGHAH *et al*., 2020; REZAC *et al*., 2018). Consequentemente, diversas cepas de BAL isoladas de diferentes substratos, como bebidas fermentadas indígenas (ALMEIDA; RACHID; SCHWAN, 2007), kefir (MAGALHÃES *et al*., 2010), cacau (PEREIRA *et al*., 2012), leite de cabra (DE ALMEIDA JÚNIOR *et al*., 2015) e linguiça suína (DIAS; SANTOS; SCHWAN, 2015) apresentam como candidatas promissoras probióticas e iniciadoras. Com isso, pesquisas têm utilizado esses isolados como culturas iniciadoras (FREIRE *et al*., 2017), além de avaliar o seu potencial probiótico (FONSECA *et al*., 2020).

BAL compreende um grupo de cocos ou bastonetes Gram-positivo, anaeróbios facultativos, geralmente não formadores de esporos, não móveis, que produzem ácido lático como o principal metabólico final da fermentação de açúcares. Os gêneros *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* e *Streptococcus* são membros importantes desse grupo (SHOKRYAZDAN *et al*., 2017). Com base no metabolismo de carboidratos e produtos finais da fermentação, BAL podem ser divididos em dois diferentes grupos: grupo homofermentativo (obrigatório) e grupo heterofermentativo, sendo este último, subdividido em espécies facultativas e obrigatoriamente fermentativas.

- Espécies homofermentativas obrigatórias, geralmente, fermentam apenas hexoses quase completamente em ácido lático pela via Embden – Meyerhof – Parnas (EMP).

- Espécies obrigatoriamente heterofermentativas degradam hexoses pela via do fosfogluconato, produzindo não apenas ácido láctico como produto final, mas também, quantidades significativas de etanol ou ácido acético e dióxido de carbono.
- Espécies facultativamente heterofermentativas degradam hexoses em ácido lático através da via EMP e, também, podem metabolizar pentoses e frequentemente gluconato, pois possuem aldolase e fosfocetolase, resultando na produção de etanol, ácido acético e ácido fórmico sobre limitação de glicose (GÄNZLE, 2015).

Além disso, espécies BAL também podem utilizar a via de fermentação malolática para aumentar a sobrevivência em condições de estresse ambiental, como baixo pH, alta concentração de carboidratos ou falta de nutrientes. Esta via envolve a descarboxilação do ácido L-málico em ácido L-lático e CO_2 (PAPADIMITRIOU *et al*., 2016).

Recentemente, o gênero *Lactobacillus* foi reclassificado devido à enorme diversidade das características fenotípicas, genotípicas e ecológicas. Com base na abordagem polifásica, as 261 espécies pertencentes ao gênero *Lactobacillus* foram reclassificadas em 25 novos gêneros, que inclui os gêneros Lactobacillus, Paralactobacillus e 23 novos gêneros (ZHENG *et al*., 2020). Juntamente com o gênero *Lactobacillus* (*L. acidophillus*), as espécies de *Lacticaseibacillus* (*L. casei*, *L. rhamnosus*), *Lactiplantibacillus* (*L. plantarum*), *Ligilactobacillus* (*L. salivarius*) e *Limosilactobacillus* (*L. fermentum*, *L. reuteri*) são as mais comumente usadas como probióticos (PIMENTEL *et al*., 2021).

Embora uma ampla variedade de gêneros e espécies de microrganismos sejam considerados probióticos em potencial, o uso comercial em alimentos probióticos são predominantemente *Lactobacillus* spp. e gêneros relacionados (TRIPATHI; GIRI, 2014). Algumas das principais cepas vendidas, seja liofilizadas, em forma de suplementos ou incorporadas em alimentos não lácteos, estão resumidas na Tabela 1.

Tabela 1 - Algumas das principais cepas probióticas utilizadas em produtos alimentícios não lácteos comercialmente disponíveis.

Produto probiótico	Empresa	Tipo	Cepa
Ingredientes	DuPont Nutrition	Liofilizada	*Lactobacillus acidophilus* NCFM
	Chr. Hansen	Liofilizada	*Lacticaseibacillus paracasei* CRL 431 (antigo *Lactobacillus paracasei* CRL 431) *Limosilactobacillus fermentum* VRI003 (antigo *Lactobacillus fermentum* VRI003 (PCC®) *Limosilactobacillus reuteri* RC-14 (antigo *Lactobacillus reuteri* RC-14) *Lacticaseibacillus rhamnosus* GR-1 (antigo *Lactobacillus rhamnosus* GR-1)
	Probi AB	Liofilizada	*Lactiplantibacillus plantarum* 299v (antigo *Lactobacillus plantarum* 299v)
Produtos alimentícios			
Yosa	Bioferme Ou	Iogurte de farelo de aveia	*Lactobacillus acidophilus* La-05
Bio K Plus	Bio K Plus International Inc.	Arroz e leite de soja	*Lactobacillus acidophilus* CL1285 *Lacticaseibacillus casei* LBC80R (antigo *Lactobacillus casei* LBC80R) *Lacticaseibacillus rhamnosus* CLR2
Suplementos/Medicação			
Sho Balance	Sho Nutrition	Géis esféricos veganos	*Lacticaseibacillus casei* K-1
Protectis	University College	Comprimidos mastigáveis ou gotas probióticas	*Ligilactobacillus salivarius* UCC118 (antigo *Lactobacillus salivarius* UCC118)

Fonte: Adaptado de Pimentel *et al*. (2021).

2.2. Critérios para seleção de microrganismos probióticos

No Brasil, o uso de probióticos em alimentos requer prévia avaliação da Anvisa, segundo requisitos da Resolução RDC Anvisa nº 241, de 27 de julho de 2018. A avaliação efetuada contempla a comprovação da identidade da linhagem do microrganismo, de sua segurança e de seu efeito benéfico (BRASIL, 2018). No entanto, existem inúmeros testes que podem ser empregados na caracterização de microrganismos probióticos, mas os seguintes, são os principais: resistência à acidez gástrica e aos ácidos biliares, aderência ao muco e/ou células epiteliais humanas e linhas celulares, atividade antimicrobiana contra bactérias potencialmente patogênicas, capacidade de reduzir a adesão de patógenos às superfícies e atividade da hidrolase do sal biliar (BYAKIKA *et al*., 2019). É importante reportar que os benefícios à saúde

mostrados para uma cepa podem não ser estabelecidos para outra cepa, mesmo da mesma espécie (SANDERS *et al*., 2018).

2.2.1. Critérios de segurança

Membros do gênero *Lactococcu*s, *Lactobacillus* (e gêneros relacionados) e algumas outras cepas BAL são geralmente reconhecidas como seguras (GRAS, por suas siglas em inglês), embora contenham alguns patógenos oportunistas (ŽUNTAR *et al*., 2020). Por isso, a comprovação da segurança da linhagem probiótica deve ser verificada um pouco além, pela identificação taxonômica do microrganismo e histórico de uso, por meio de ensaios *in vitro*, como padrões de resistência a antibióticos, atividades metabólicas (produção de D-lactato, desconjugação de sais biliares), avaliação de efeitos colaterais em humanos, vigilância epidemiológica de incidentes adversos em consumidores, produção de toxinas (para cepas que pertencem a uma espécie conhecida pela produção de toxinas) e atividade hemolítica para cepas que pertencem a uma espécie conhecida por hemólise (BYAKIKA *et al*., 2019). Estudos recentes têm avaliado a segurança de cepas potencialmente probióticas, por meio da produção de enzimas hidrolíticas específicas, por exemplo, gelatinase e DNAse (DIVISEKERA *et al*., 2019; FONSECA *et al*., 2020; PINTO *et al*., 2020).

Como a maioria dos probióticos são membros comuns do TGI humano ou são ingeridos em grandes quantidades, a presença de determinantes de resistência a antibióticos em seu genoma merece atenção especial, devido à possibilidade de transferência de genes (BINDA *et al*., 2020). Os lactobacilos não têm fatores de virulência, devido aos quais, as infecções raramente foram relatadas em humanos saudáveis. No entanto, a avaliação de segurança é um critério importante para qualquer microrganismo de qualidade alimentar, especialmente no caso de novas cepas que não têm histórico de uso seguro (PRADHAN; MALLAPPA; GROVER, 2020).

Portanto, a avaliação individual da segurança das cepas deverá ser considerada, as quais precisam cumprir os requisitos de segurança estipulados pelo órgão regulador (BINDA *et al*., 2020). Além disso, estudos de segurança *in vivo* em modelos animais, como toxicidade aguda, subaguda e crônica da ingestão de quantidades extremamente grandes de probióticos, não apenas em animais saudáveis, mas também em animais experimentais doentes, devem ser realizados antes do início de novos ensaios clínicos em humanos (PRADHAN; MALLAPPA; GROVER, 2020).

2.2.2. Características funcionais

A microbiota intestinal humana é fundamental para a manutenção da saúde, ao mesmo tempo que uma microbiota disbiótica está associada a diversas doenças. Os efeitos terapêuticos e profiláticos de alguns probióticos contra distúrbios relacionados ao intestino podem ser, pelo menos em parte, mediados pela modificação da microbiota e/ou de sua função. Os microrganismos probióticos agem através de uma variedade de meios, incluindo imunomodulação, produção de ácidos orgânicos e compostos antimicrobianos, interação com a microbiota residente, interface com o hospedeiro, melhorando a integridade da barreira intestinal e a produção de enzimas (SANDERS *et al*., 2019). Diversas propriedades probióticas podem ser exercidas por diferentes cepas BAL. Efeitos imunomodulador, antioxidante, antimutagênico, anti-infecção, antiviral, anti-inflamatório e balanço da microbiota intestinal são algumas das recentes propriedades documentadas (KHANEGHAH *et al*., 2020).

Os resultados dos estudos clínicos confirmam o efeito positivo dos probióticos em doenças gastrointestinais (por exemplo, síndrome do intestino irritável, distúrbios gastrointestinais, eliminação de *Helicobacter*, doença inflamatória do intestino, diarreias) e doenças alérgicas (por exemplo, dermatite atópica). Muitos estudos clínicos comprovaram a eficácia dos probióticos no tratamento de doenças como obesidade, síndrome de resistência à insulina, diabetes tipo 2 e doença hepática gordurosa não alcoólica. Além disso, os efeitos positivos dos probióticos na saúde humana foram demonstrados pelo aumento da imunidade do corpo (imunomodulação). Relatórios científicos também mostram os benefícios do uso profilático de probióticos em diferentes tipos de câncer e os efeitos colaterais associados ao câncer (MARKOWIAK; ŚLIZEWSKA, 2017). Ainda no intestino humano, as bactérias *Leuconostoc*, *Bifidobacterium*, *Lactobacillus* e gêneros relacionados crescem bem e impedem o crescimento de patógenos intestinais desfavoráveis. Estes microrganismos podem ser usados individualmente ou em combinação, para melhorar o metabolismo da lactose, prevenir infecção do trato intestinal, reduzir o colesterol sérico, sintetizar vitaminas, ácidos nicotínicos e ácidos fólicos, estimular a absorção de cálcio, melhorar a digestibilidade das proteínas, controlar efeitos danosos de patógenos de origem alimentar, reduzir a hipercolesterolemia, doenças cardiovasculares e pancreatite (PANGHAL *et al*., 2018).

Os estudos moleculares e genéticos permitiram determinar os fundamentos do efeito benéfico dos probióticos, envolvendo quatro mecanismos, de acordo com Markowiak e Ślizewska (2017):

(1) Antagonismo através da produção de substâncias antimicrobianas;

(2) Competição com patógenos pela adesão ao epitélio e por nutrientes;

(3) Imunomodulação do hospedeiro;

(4) Inibição da produção de toxinas bacterianas

Finalmente, as bactérias probióticas têm a capacidade de produzir uma ampla gama de metabólitos com benefícios para a saúde dos humanos. Os compostos bioativos produzidos por bactérias probióticas incluem, por exemplo, bacteriocinas, enzimas metabólicas, aminoácidos e peptídeos, ácidos graxos de cadeia curta, vitaminas, antioxidantes e exopolissacarídeos. Coletivamente, essas moléculas aumentam a função fisiológica do intestino e melhoram a saúde (CHUGH; KAMAL-ELDIN, 2020).

2.2.3. Propriedades tecnológicas

A melhoraria da viabilidade dos probióticos em diferentes produtos alimentícios durante sua produção até o momento do consumo requer exaustivos estudos. Muitos fatores foram relatados por influenciar a viabilidade de microrganismos probióticos em produtos alimentícios durante a produção, processamento e armazenamento. Os fatores identificados incluem parâmetros dos alimentos (pH, acidez titulável, oxigênio molecular, atividade de água, presença de sal, açúcar e produtos químicos, como peróxido de hidrogênio, bacteriocinas, aromatizantes e corantes artificiais); parâmetros de processamento (tratamento térmico, temperatura de incubação, taxa de resfriamento do produto, materiais de embalagem, métodos de armazenamento e escala de produção); e parâmetros microbiológicos (cepas de probióticos, taxa e proporção de inoculação) (TRIPATHI; GIRI, 2014).

Uma das propriedades tecnológicas essenciais é a capacidade de uma cepa ser cultivada em grande escala. Para novas cepas, é importante ter tanto chances razoáveis de aumento de escala industrial e sobrevivência, quanto sucesso em encontrar os benefícios de saúde desejados. Além disso, uma cepa probiótica também deve apresentar boas propriedades sensoriais e não alterar a textura ou o sabor de um produto (FORSSTEN; SINDELAR; OUWEHAND, 2011).

Os probióticos têm que sobreviver aos diferentes processos de produção, com diferentes níveis de atividade de água, pH, teor de oxigênio e temperaturas. O pH é um dos fatores mais importantes que afetam a sobrevivência dos probióticos. Os sucos contêm alto teor de ácidos orgânicos e o baixo pH aumenta a concentração da forma não dissociada. Portanto, em sucos, poderíamos presumir a existência de uma ação combinada de condições ácidas e do efeito antimicrobiano intrínseco dos ácidos. *Lactobacillus* e gêneros associados são, geralmente, resistentes e sobrevivem em sucos com pH variando de 3,7 a 4,3 (PERRICONE *et al*., 2015). Outros fatores a serem considerados, incluem a concentração de oxigênio no ambiente de fermentação, relacionada diretamente à natureza homo ou heterofermentativa das culturas, à escolha de substratos, particularmente os tipos e concentrações de açucares e o tratamento da matéria-prima por homogeneização, por exemplo, pode permitir um metabolismo mais eficaz e liberar peptídeos bioativos. O tempo e a intensidade do calor, durante a pasteurização, também precisam ser considerados (MARSH *et al*., 2014).

Para produtos onde o probiótico está se metabolizando ativamente, a estabilidade pode ser uma função das capacidades inerentes da bactéria, bem como das propriedades físicas da matriz alimentar. A estabilidade não está ligada às características de um gênero ou espécie particular, embora existam semelhanças típicas. No entanto, a sobrevivência exata está ligada a uma cepa específica (FORSSTEN; SINDELAR; OUWEHAND, 2011).

É importante revisar não apenas a boa sobrevivência dos probióticos, mas também, a aceitação sensorial dos alimentos probióticos não lácteos durante a produção e o armazenamento. As propriedades sensoriais de alimentos probióticos não lácteos podem ser afetadas por interações de diferentes espécies de probióticos e matrizes alimentares, onde texturas, sabor, aroma e cor, por exemplo, podem ser melhorados ou piorados pela produção de diferentes compostos metabólicos. Os sucos de frutas receberam muito interesse como potenciais alimentos não lácteos transportadores de probióticos, mas algumas características sensoriais indesejáveis, como sabor estranho, sabor e gosto residual ácidos, são observadas, principalmente, neste tipo de alimento (MIN *et al*., 2019). No entanto, pesquisas atuais reportaram que sucos de frutas fermentados por BAL apresentaram, em relação à aceitação geral, pontuações que os classificam como produtos aceitáveis do ponto de vista sensorial, ou seja, média acima de 6,0 na escala hedônica no teste de aceitabilidade (HASHEMI; JAFARPOUR, 2020; WU *et al*., 2021).

2.3. Desenvolvimento de novos alimentos probióticos

A mudança dos hábitos alimentares e a conscientização sobre a saúde têm levado os consumidores à procura de alimentos mais nutritivos, saudáveis, relacionados ao bem-estar e que trazem benefícios para a saúde; principalmente, na prevenção de doenças crônicas não transmissíveis (PANGHAL *et al*., 2018). Embora diversas BAL funcionais tenham sido aplicadas em alimentos probióticos fermentados em todo o mundo, o mercado de produtos biofuncionais está, continuamente, precisando da implementação e diversificação dos produtos disponíveis (PEREIRA *et al*., 2018). Sendo assim, a combinação de probióticos com frutas pode fornecer, simultaneamente, os probióticos e os nutrientes de que o corpo necessita, indicando uma importante direção de desenvolvimento para a indústria de probióticos no futuro. A fermentação pode produzir ácidos orgânicos, aminoácidos e vários compostos aromáticos que podem intensificar o sabor desejável dos produtos. Além disso, grandes quantidades de substâncias ativas como ácidos graxos de cadeia curta, polissacarídeos viscosos e peptídeos são produzidas por fermentação, o que pode reduzir a constipação, aliviar a colite e prevenir e tratar a inflamação do trato digestivo (GUAN; XIONG; XIE, 2021).

Atualmente, sucos comercialmente probióticos são propostos por muitos fabricantes, verificando o alto interesse e importância desses novos produtos. Vários sucos foram aplicados isoladamente ou misturados com outros sucos e muitas bactérias probióticas, principalmente BAL, foram usadas na respectiva fermentação ácido-lática dos sucos (KAZAKOS; MANTZOURANI; PLESSAS, 2020).

Pesquisas sobre produtos probióticos baseados em frutas, vegetais, cereais e leguminosas, incluindo seus benefícios para a saúde, têm sido realizadas, buscando assim, produtos alternativos e com maior aceitação pelos consumidores (PANGHAL *et al*., 2018). Em geral, os alimentos fermentados estão associados à preocupação dos consumidores com a abordagem nutricional da dieta (vitaminas, prebiótico, probiótico e digestibilidade), segurança alimentar, modificação organoléptica do alimento com novo aroma, textura ou sabor, extensão da vida de prateleira, simplicidade de preparação, reaproveitamento de matérias-primas e desenvolvimento sustentável (SEPTEMBRE-MALATERRE; REMIZE; POUCHERET, 2018).

Em frutas e vegetais, as moléculas de interesse nutricional são fibras, vitaminas, minerais, compostos fenólicos, incluindo flavonóides, fitoestrógenos, compostos de enxofre, monoterpenos e peptídeos bioativos (SEPTEMBRE-MALATERRE; REMIZE; POUCHERET, 2018). No entanto, existem algumas desvantagens e limites no uso desses substratos na produção de alimentos probióticos, como a estabilidade dos microrganismos durante o

armazenamento e os impactos nas características sensoriais e na aceitação geral do produto (PERRICONE *et al*., 2015). Desta forma, avaliações de novas matrizes vegetais para o aumento do número de produtos fermentados probióticos são essenciais.

2.4. Sucos fermentados probióticos

Entre as tecnologias de processamento de alimentos, a fermentação visa aumentar a vida útil e desenvolver características sensoriais agradáveis, modificando a composição do produto. Além disso, o interesse humano na fermentação ainda se baseia nas vantagens de melhores propriedades nutricionais, de saúde e produção de princípios ativos de interesse (SEPTEMBRE-MALATERRE; REMIZE; POUCHERET, 2018). Neste contexto, os sucos de frutas representam uma alternativa interessante como veículo de probióticos, devido ao alto teor nutricional, antioxidantes, presença de açúcares que favorecem o crescimento do microrganismo e sabores atraentes gerados pelo processo fermentativo (MARSH *et al*., 2014).

Atualmente, diversas pesquisas têm avaliado a influência da fermentação por BAL probióticas e potencialmente probióticas nas características de sucos de frutas, conforme demonstrado na Tabela 2.

Tabela 2 - Fermentação de sucos de frutas por bactérias probióticas e potencialmente probióticas.

Cepa BAL	**Fruta do suco**	**Referência**
Potencialmente probióticas		
L. plantarum BNCC 337796	Amora e mirtilo	Wu *et al*. (2021)
L. paracasei SP5	Arônia	Bontsidis *et al*. (2021)
Levilactobacillus brevis F064A (antigo *Lactobacillus brevis* F064A) Probióticas	Amora	Kanklai *et al*. (2021)
L. rhamnosus GG	Juçara e abacaxi	Campos *et al*. (2019)
L. plantarum ATCC 14917	Romã	Mantzourani *et al*. (2019)
L. paracasei K5	Romã	Mantzourani *et al*. (2020)

Fonte: Do autor (2021)

O processo de fermentação do suco de amora por espécies de *Lactobacillus* e gêneros associados melhorou significativamente na concentração total de antocianinas, compostos fenólicos, flavonoides e atividade antioxidante (KWAW *et al*., 2018). Outros estudos

reportaram benefícios relacionados à fermentação de sucos de frutas, como o aumento da viabilidade celular e atividade antibacteriana contra *S.* Typhimurium e *E. coli* O157:H7 em suco de limão fermentado por *L. plantarum* LS5 (HASHEMI *et al*., 2017). Considerando os compostos voláteis, Di Cagno, Filannino e Gobbetti (2017) relataram mudanças no perfil aromático do suco de romã após a fermentação, e que o processo contribuiu com atributos sensoriais positivos, como notas de floral e frutado, além de limitar compostos indesejáveis.

No mesmo sentido, Zhao *et al*. (2019) observaram maior aceitação global do suco de jujuba fermentado por culturas mistas de *Lactobacillus*, quando comparado com o suco não fermentado. De acordo com Pimentel *et al*. (2021), os principais impactos promovidos por cepas probióticas nos parâmetros tecnológicos e sensoriais de sucos de frutas são: alterações positivas na composição química, maior acidez, redução nos sólidos solúveis totais, alterações na turbidez, aumento do teor de compostos fenólicos, aumento do teor de antocianinas e flavonóides e aceitação sensorial semelhante ou reduzida.

Em suma, a escolha e estudos do processo ideal é crucial para alcançar alta viabilidade celular e, com isso, garantir que os consumidores obtenham os benefícios associados à saúde (LU; PUTRA; LIU, 2018). Finalmente, o desenvolvimento de produtos probióticos deve considerar a fonte da cepa, o impacto das etapas de processamento, matriz alimentar, forma de adição de probióticos, efeitos específicos da cepa, condições de armazenamento e adição de componentes prebióticos, portanto, produtos com alta qualidade podem ser obtidos (PIMENTEL *et al*., 2021).

3. CONSIDERAÇÕES GERAIS

A seleção e caracterização de novas espécies e cepas mais específicas de bactérias probióticas estão oferecendo novas oportunidades para a inovação no setor de probióticos. Em geral, os critérios de seleção estabelecidos incluem propriedades funcionais, tecnológicas e de segurança. Apesar dos vários benefícios à saúde do hospedeiro relacionados a esses microrganismos, a prevenção de infecções do trato intestinal merece atenção e estudo, pois são recorrentes as doenças provocadas por patógenos de origem alimentar.

Adicionalmente, após a seleção de cepas com destacáveis propriedades benéficas, é importante avaliar suas características tecnológicas. Para isso, a avaliação da capacidade de crescer em altas concentrações no substrato utilizado, manter a estabilidade durante o armazenamento e produzir características sensoriais agradáveis aos consumidores são considerados os maiores desafios para a indústria de probióticos não lácteos.

Neste sentido, a fermentação de matrizes não lácteas a base de frutas desperta o interesse humano, uma vez que recomendações nutricionais que enfatizam o consumo destes produtos estão relacionadas a benefícios para a saúde associados a moléculas presentes nestes alimentos, além das vantagens do processo fermentativo em aumentar a segurança e a vida útil e produzir modificações organolépticas diferenciadas e únicas ao produto.

Por fim, sugestões para trabalhos futuros incluem testes *in vivo* e clínicos para confirmar as propriedades da cepa selecionada e avaliação de diferentes características alternativas, como atividades anticolesterol, antiansiedade, anti-inflamatória, entre outros. Além disso, a utilização das ferramentas “ômicas”, como genômica, proteômica, metabolômica e transcriptômica podem ser usadas para avaliar a expressão de genes ou mesmo identificar modificações no metabolismo microbiano em diferentes condições relacionadas ao hospedeiro.

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SEGUNDA PARTE - ARTIGOS

ARTIGO 1

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Probiotic Properties of Lactobacilli and Their Ability to Inhibit the Adhesion of Enteropathogenic Bacteria to Caco-2 and HT-29 Cells

Abstract

We evaluated the probiotic properties of lactic acid bacteria using resistance, safety, and functional assays. A preliminary subtractive screening of nineteen strains was performed based on their survival in simulated gastric and intestinal juice, and cell surface characteristics (hydrophobicity and auto-aggregation). Five strains were selected for further characterization, which included the assessment of their co-aggregation to pathogens, phenol tolerance, antimicrobial activity, and safety. Moreover, their adhesion to Caco-2 and HT-29 cells and the ability to inhibit pathogenic bacteria adhesion were evaluated. All strains had high (≥80.0%) survival rates in gastric and intestinal juices. Amongst them, *Lactobacillus brevis* CCMA 1284, *L. plantarum* CCMA 0743, and *L. plantarum* CCMA 0359 exhibited higher hydrophobicity (95.33, 96.06, and 80.02%, respectively), while *L. paracasei* CCMA 0504 and *L. paracasei* CCMA 0505 had the highest auto-aggregation values (45.36 and 52.66%, respectively). However, these last two strains were positive for the DNAse test, which is a safety concern. The CCMA 0359 and CCMA 1284 strains did not show antimicrobial activity, while the CCMA 0505 strain had a higher percentage of adhesion (4.75%) to Caco-2 cells. In the simulated competition and exclusion assays, the CCMA 0743 strain was able to reduce *Salmonella* adhesion to both cells (Caco-2 and HT-29), but only the CCMA 0743 and CCMA 0505 strains inhibited *Escherichia coli* adhesion to HT-29 cells in the competition assay. According to the results of these evaluated attributes, this strain showed to be an excellent candidate for probiotic use.

Keywords

Antagonistic effects, Brazilian foods, Enteropathogenic *Escherichia coli*, Epithelial cells, Gastrointestinal tract, *Salmonella* Enteritidis

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Compliance with Ethical Standards

Conflict of Interest

The authors declare that they have no conflict of interest.

Introduction

Probiotic microorganisms are recognized for their many health benefits. Among the known probiotic microorganisms, various species of lactic acid bacteria (LAB), especially those of the *Lactobacillus* genus, are widely used as probiotic cultures, as well as for the development of probiotic fermented products [1, 2]. Although there are several strains with proven probiotic properties on the market, the search for novel strains with functional and technological characteristics remains an attractive goal to satisfy increasingly demanding consumers [1, 2], contributing to improved health and reducing the risk of disease [3].

LAB can be found in a variety of food matrices such as dairy products [4], meats [5], indigenous fermented beverages [6], and cocoa [7]. Thus, these food sources are potential reservoirs of novel probiotic strains. The study and selection of new probiotic strains require a systematic approach consisting of sequential evaluations to reduce the number of candidate strains. Probiotic characteristics are reported as strain-specific [8]; therefore, the evaluation of both wild and novel strains is essential, since isolates belonging to the same species may display different properties and probiotic mechanisms.

The methods and criteria used to characterize probiotic strains include assessing their ability to tolerate stress conditions exerted by the human body, ability to interact with host epithelial cells, safety attributes (such as β-hemolysis, gelatinase, and DNAse enzyme activities), and sensitivity to antibiotics [9], antimicrobial activity, and competition with pathogens [10]. Moreover, cell surface properties (hydrophobicity, autoaggregation, and co-aggregation with pathogens) and the interaction of candidate strains with human epithelial cell lines and pathogenic bacteria represent different mechanisms that can be considered in the evaluation of probiotic efficacy [11, 12]. *In vitro* models employing the HT-29 and Caco-2 cell lines isolated from colonic adenocarcinomas have been widely used to investigate probiotic adhesion capacity [13].

Enteropathogenic *Escherichia coli* (EPEC) and *Salmonella enterica* are important human pathogens whose virulence traits depend on their ability to adhere to epithelial intestinal cells [14, 15]. The indiscriminate use of antibiotics to combat these microorganisms has contributed to the development of resistance mechanisms [16]. In this sense, probiotics have emerged as an alternative in the treatment of bacterial infections, mainly due to the protection conferred to the host cells [17]. Some studies have indicated that the LAB may prevent or reduce the attachment of pathogens to host cells [17, 18]. Here we evaluated the *in vitro* probiotic properties of wild LAB strains isolated from different fermented food products. The antagonistic effects of selected LAB strain on the adhesion of pathogens to Caco-2 and HT-29 cells were evaluated by exclusion and competition assays.

Materials and Methods

Screening of LAB Strains

Nineteen LAB strains belonging to the Culture Collection of Agricultural Microbiology (CCMA) of the Federal University of Lavras and isolated from different substrates were initially employed in this study (Table 1). Sequentially, the pre-selection of LAB cultures was based on their ability to survive simulated gastric and intestinal juices and cell surface characteristics (hydrophobicity and auto-aggregation). Five strains (three with higher hydrophobicity and two with higher auto-aggregation) were selected for further characterization.

Table 1 LAB strains obtained from CCMA

Specie	Code	Source	Reference
Lactobacillus paracasei	CCMA 0504	Apple juice kefir	[19]
Lactobacillus paracasei	CCMA 0505	Apple juice kefir	[19]
Lactobacillus paracasei	CCMA 0506	Apple juice kefir	[19]
Lactobacillus paracasei	CCMA 0354	Goat milk	[4, 20]
Lactobacillus brevis	CCMA 0355	Goat milk	[4, 20]
Lactobacillus brevis	CCMA 0351	Goat milk	[4, 20]
Lactobacillus brevis	CCMA 1284	Cauim	[21]
Lactobacillus plantarum	CCMA 0743	Cauim	[22]
Lactobacillus plantarum	CCMA 0361	Cocoa	[8]
Lactobacillus plantarum	CCMA 0359	Cocoa	[8]
Enterococcus faecium	CCMA 0416	Rice chicha	[23]
Enterococcus faecium	CCMA 0418	Rice chicha	[23]
Lactobacillus casei	CCMA 0412	Rice chicha	[23]
Lactobacillus casei	CCMA 0411	Rice chicha	[23]
Lactobacillus casei	CCMA 0784	Corn silage	[24]
Lactobacillus rhamnosus	CCMA 1431	Corn silage	[24]
Lactobacillus acidophilus	CCMA 0779	Corn silage	[24]
Lactobacillus fermentum	CCMA 0201	Yakupa	[25]
Lactobacillus fermentum	CCMA 0203	Yakupa	[25]

Survival to Simulated Gastric and Intestinal Juices

The survival of LAB in simulated gastric and intestinal juices was assessed as previously described [26] with modifications. Cultures were grown for 16 h at 37 °C in sterile MRS both, then 1mL of each culture was mixed in 9 mL sterile saline solution (NaCl 0.85% w/v) with pH adjusted to 2.0 using 1 M hydrochloric acid (HCl) containing 0.5% pepsin (Fisher Scientific, UK) (w/v). After mixing, the initial bacteria counts were determined by plating. Subsequently, samples were incubated for 90 min at 37 °C and cell viability determined by plating. Then, the simulated intestinal juice was prepared by adding oxgall (Himedia, Mumbai, India) and pancreatin (Dinâmica, Brazil) solutions to obtain final concentrations of 0.3% and 0.1% (w/v), respectively, and the pH was then adjusted to 7.0 by adding 1 M sodium hydroxide (NaOH). After mixing, samples were incubated at 37 °C for 150 min, then viable cell counts were determined. All sample counts were determined by plating on MRS (Man Rogosa and

Shape, Kasvi, Italy) agar. The experiments were repeated three times and performed in triplicate. Results were expressed as mean log colony-forming units per mL (CFU/mL). The survival rate was calculated as follows:

$$\text{Survival } (\%) = \left[\text{final}\left(\text{Log CFU/mL}\right) / \text{initial}\left(\text{Log CFU/mL}\right)\right] \times 100$$

Determination of Cell Surface Characteristics

The hydrophobicity, auto-aggregation, and co-aggregation assays were performed according to Kaktcham et al. [27] with slight modifications.

Hydrophobicity

The cell surface hydrophobicity of each strain was assessed by measuring microbial affinity to xylene. Briefly, cells collected from a 16 h old culture were centrifuged (10000 rpm for10 min). The resulting pellet was washed twice with sterile phosphate-buffered saline (PBS) (pH=7.2) and re-suspended in the same buffer. The optical density at the 600 nm wavelength (OD_{600}) of the suspension was measured (A0) using a spectrophotometer (Spectrum, SP-2000UV). Thereafter, 1 mL of xylene was added to 3 mL of cell suspension and mixed by vortexing for 2 min. Then, the water and xylene phases were separated by incubation for 1 h at 37 °C. The aqueous phase was removed and the new OD_{600} was measured (A1). The percentage of the cell surface hydrophobicity was calculated using the formula:

Hydrophobicity (%) = (1- A1/A0) x 100

The strains were then classified into microorganisms of low (0-34%), moderate (35-69%) and high hydrophobicity (70-100%) [28].

Auto-aggregation

LAB cells were harvested from a 16 h old culture in MRS broth, washed twice with PBS (pH 7.2), re-suspended in the same buffer, and diluted to an OD_{600} of 0.6 ± 0.1 (approximately 7-8 Log CFU/mL). Bacterial cell suspensions were vortexed for 10 s and subsequently incubated at 37 °C for 5 h. The auto-aggregation percentage was determined using the equation:

$$\text{Auto-aggregation}\left(\%\right) = \left(1 - \text{At} / \text{A0}\right) \times 100$$

where At represents the absorbance at time t = 5 h and A0 represents the absorbance at time t = 0 h.

Co-aggregation

For co-aggregation, the LAB strains were grown in MRS broth for 16 h at 37 °C, while enteropathogenic *Escherichia coli* (EPEC) CDC 055 and *Salmonella enterica* serovar Enteritidis ATCC 564 were grown in BHI

(Brain-Heart Infusion) broth for 24 h at 37 °C. Bacterial suspensions were prepared as described in the auto-aggregation test above. Equal volumes (2 mL) of LAB and human pathogen suspensions were mixed by vortexing (10 s) and incubated at room temperature without agitation for 4 h. Control tubes contained 2 mL of the suspension of each bacterial strain. The absorbances (OD_{600}) of the mixtures and controls were measured after incubation. The percentage of co-aggregation was calculated using the following formula:

$$\text{Co-aggregation}(\%) = \left[\left(A_{lab} + A_{pat}\right) - 2A_{mix} / \left(A_{lab} + A_{pat}\right)\right] \times 100$$

where A_{lab} and A_{pat} refer to the OD_{600} of the LAB cell suspension and pathogen cell suspension, respectively, in control tubes and A_{mix} represents the absorbance of the mixed bacterial suspension tested after 4 h.

Phenol Tolerance

Phenol tolerance was determined according to the method described by Shehata et al. [29]. Overnight cultures of LAB strains were inoculated (1%) into MRS broth with 0.2 and 0.5% (v/v) of phenol, or without phenol. Bacterial cells in the culture broth were quantified by reading the OD_{600} after 24 h of incubation at 37 °C. The experiments were performed in duplicate.

Antimicrobial activity

The antimicrobial activity of LAB cultures was evaluated by the agar spot test according to Arena et al. [30] with modifications. An aliquot of 5 µL of each LAB isolate previously grown in MRS broth was separately spotted on MRS agar and plates were incubated at 37 °C for 48 h to allow the expression and secretion of antimicrobial compounds produced by cultures. The indicator microorganisms were *Salmonella enterica* serovar Enteritidis ATCC 564, enteropathogenic *Escherichia coli* (EPEC) CDC 055, *Listeria monocytogenes* ATCC 19117, *Staphylococcus aureus* ATCC 5674, and *Bacillus cereus* ATCC 14579. Overnight cultures of indicator microorganisms were mixed 1:100 with BHI soft agar (0.7% w/v) and overlaid on developed colonies (8 mm diameter) of LAB isolates. After incubation at 37 °C for 24 h, plates were checked for zones of inhibition surrounding the producer colonies. The experiment was repeated three times.

Antibiotic Susceptibility

The antibiotic susceptibility of the strains was determined by the disk diffusion assay. Overnight cultures (100 µL) were spread onto MRS agar media and antibiotic discs containing ampicillin (10 µg), vancomycin (30 µg), gentamicin (10 µg), streptomycin (10 µg), chloramphenicol (30 µg), erythromycin (15 µg), azithromycin (15 µg), penicillin (10 µg), novobiocin (30 µg), oxacillin (1 µg), and lincomycin (2 µg) were placed on the surface of the inoculated plates using sterile forceps. Inhibition zone diameters were measured after incubation at 37 °C for 24 h. The susceptibility of the isolates was categorized as resistant (R), moderately susceptible (MS) or susceptible (S) according to interpretative values [31]. The experiment was repeated three times.

In Vitro Assessment of Safety Attributes

The safety of the isolates was investigated by assessing hemolysis, DNAse activity, and gelatin hydrolysis, as described by Singh et al. [32] with modifications. Hemolytic activity was determined by inoculating the strains on blood agar plates containing 5% sheep blood after 48 h incubation at 37 °C. The absence of an effect on blood plaques (γ-hemolysis) was considered non-hemolytic. Green-hued zones around the colonies (α-hemolysis) were considered as partial hemolytic activity and strains showing clear areas of hydrolysis resulting from blood cell lysis around the colonies were classified as hemolytic strains (ß-hemolysis). Gelatinase production by strains was analyzed using tryptone-neopeptone-dextrose (TND) agar (17.0 g tryptone, 3.0 g neopeptone, 2.5g dextrose, 5.0 g NaCl, 2.5g K_2HPO_4, 15 g agar, and 1 L distilled water) containing 0.4% gelatin. The LAB cultures were spot-inoculated onto plates containing the medium and incubated at 37 °C for 48 h. Enzyme production was visualized by the formation of a halo around the colony after addition to a saturated ammonium sulfate solution to confirm gelatin hydrolysis. For the DNAse test, strains were streaked on the DNAse test agar medium (Difco, USA) and the plates were incubated at 37 °C for 48 h. After this time, a 1 M HCl solution was added to the plate. A clear zone around the colonies after incubation was considered positive for DNAse production. For all tests, the *Staphylococcus aureus* ATCC 25923 strain was used as the positive control. The experiment was repeated three times.

Adhesion of LAB Strains to Caco-2 and HT-29 Cell Lines

Growth and maintenance of Caco-2 and HT-29 cells

The Caco-2 and HT-29 cells provided by the Cell Bank of Rio de Janeiro (BCRJ, Rio de Janeiro) were grown in modified Eagle's minimal essential medium (MEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum), 1x non-essential amino acids, 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. The culture medium was changed regularly and when the cells reached sub-confluence (80-90%), they were sub-passaged.

Adhesion assay on Caco-2 and HT-29 cells

The adhesion capacity test for the five selected strains (based on previous assays) to the human colon adenocarcinoma cell lines (Caco-2 and HT-29) was performed according to Ramos et al. [8] with slight modifications. The Caco-2 and HT-29 cells were sub-cultured (2 x 10^5 cells/mL) in 24-well tissue culture plates (Sarstedt, Germany) and grown at 37 °C in a humidified atmosphere of 5% CO_2 for 21 days to promote differentiation in cell media. The culture medium was changed on alternate days.

For the adhesion assay, bacteria were cultured in MRS broth for 16 h at 37 °C and after washing twice with the phosphate-buffered solution (PBS), the cultures were resuspended in the media (described above) at a concentration of approximately 10^8 CFU/mL. One milliliter of each bacteria suspension was added to cells in each well and incubated for 90 min at 37 °C in a 5% CO_2 atmosphere. Subsequently, the cells were washed three times with 1 mL of PBS to remove non-adherent bacteria cells and then lysed with 1 mL of Triton-X solution (0.1% v/v in PBS). After 10 min of incubation at 37 °C, the solution with released bacteria cells was serially diluted and plated on MRS agar. The plates were incubated at 37 °C for 48 h. Adhesion ability was expressed as the percentage ratio between the initial counts of bacteria seeded and the counts after the washing steps (CFU/mL). Experiments

were performed in duplicate and repeated three times. The probiotic strain *L. paracasei* LBC-81 (Danisco A/S, Copenhagen, Denmark) was employed as a reference strain.

Inhibition of pathogenic bacteria adhesion to Caco-2 and HT-29 cells

Cell cultures were maintained as previously described. For the pathogen adhesion inhibition test, two different types of experiments were performed using modifications to previously described procedures [33]. In the competition assay, lactobacilli suspensions (10^8 CFU/mL) and *Salmonella* or *E. coli* (10^8 CFU/mL) were mixed and co-cultured simultaneously for 90 min with Caco-2 and HT-29 monolayers. In the exclusion assay, Caco-2 and HT-29 cells were first preincubated with lactobacilli suspensions (10^8 CFU/mL) for 30 min and then a *Salmonella* or *E. coli* suspension (10^8 CFU/mL) was added to each well. Cell cultures in the presence of bacteria were incubated for an additional 90 min at 37 °C in a 5% CO_2 atmosphere. Afterward, the cells were washed three times with 1 mL of PBS to remove non-adherent bacteria cells and lysed with 1 mL of Triton-X solution (0.1% v/v in PBS). After 10 min of incubation at 37 °C, the solution of released bacteria cells was spread on BHI agar and the plates were incubated at 37 °C for 16 h. After this time, enumerations of pathogen colonies were performed. Experiments were performed in duplicate and repeated three times.

Statistical Analysis

Data were analyzed by one-way analysis of variance, followed by post-hoc Tukey's and Dunnett's tests for multiple comparisons. Differences were considered statistically significant when $p < 0.05$. All statistical analysis was carried out using Statistica software version 10.0 (Statsoft, USA).

Results and Discussion

Survival in Simulated Gastric and Intestinal Juices and Cell Surface Properties

Eighteen isolates had survival rates ≥ 81% after exposure to simulated gastric and intestinal juices, except *L. casei* CCMA 0411 that showed a survival rate of 79.42% (Table 2). The LAB strains evaluated in the present study exhibited variable hydrophobicity values ranging from 6.67 to 96.06% (Table 2). On the other hand, approximately 68% (13) of the strains had low hydrophobicity (< 35.0%). According to Kaktcham et al. [27], the composition of the bacterial membrane influences the hydrophobicity of the cell surface and, therefore, hydrophobicity evaluation is important in estimating the ability of strains to adhere to host epithelial cells. Regarding auto-aggregation capacity, the strains had values ranging from 16.50 to 52.66% after 5 h of incubation. The highest values were found for *L. paracasei* strains (CCMA 0504 and CCMA 0505) which exhibited auto-aggregation in the range of 41.00 - 60.00% (Table 2). This is important because auto-aggregation allows the formation of a barrier that prevents the colonization of pathogens on surfaces of the mucosa [34].

Table 2 Number of LAB strains able to survival to in vitro GIT conditions, and hydrophobicity and auto-aggregation capacity of the strains distributed according to percentage values

The survival rate, hydrophobicity or autoaggregation (%)	**N° of isolates (n=19)**		
	GIT Survival rate	**Hydrophobicity**	**Autoaggregation**

81 - 100	18	2	
61 – 80	1	1	
41 – 60		2	2
21 – 40		9	15
0 – 20		5	2

Based on the results obtained for survival in simulated gastric and intestinal juices as well as assessments of hydrophobicity and auto-aggregation, five LAB strains were selected (Table 3 and 4). The selected strains showed high percentages (90.06 - 96.50%) of survival in simulated gastric and intestinal juices, indicating that they were able to tolerate stressful conditions imposed by GIT (Table 3).

Table 3 Survival of selected LAB strains under simulated gastric and intestinal juices conditions at 37 °C

Strains	**Survival to gastric and intestinal juices**			
	Initial Mean Count*	**Survival after 90 min***	**Survival after 240 min***	**Surviving percentage (%)****
L. brevis CCMA 1284	8.64 ±0.44	8.62 ±0.06	8.19 ±0.01	95.04
L. plantarum CCMA 0743	8.37 ±0.12	7.97 ±0.01	8.08 ±0.06	96.50
L. plantarum CCMA 0359	8.36 ±0.35	7.73 ±0.08	8.02 ±0.01	96.15
L. paracasei CCMA 0504	7.05 ±0.05	6.84 ±0.36	6.36 ±0.84	90.06
L. paracasei CCMA 0505	7.17 ±0.75	6.82 ±0.15	6.54 ±0.66	90.94

*The values are reported as Log CFU/mL (mean of 3 experiments, each was carried out in triplicate ± Standard Error).
**No significant difference ($p>0.05$), according Tukey test.

Researchers have suggested a correlation between hydrophobicity and aggregation capacity [35, 36]. All of the five selected strains, which exhibited hydrophobicity, also displayed auto-aggregation capacities after 5 h of incubation (Table 4). All selected strains were able to co-aggregate with EPEC and *S.* Enteritidis, except *L. paracasei* CCMA 0504, which did not co-aggregate with *S.* Enteritidis (Table 4). The co-aggregation abilities of *Lactobacillus* strains can prevent intestinal colonization by pathogenic bacteria and represent an important host defense mechanism [37, 38].

Table 4 Percentage of hydrophobicity, auto-aggregation, and co-aggregation of selected LAB strains

Strains	**Hydrophobicity**	**Auto-aggregation**	**Co-aggregation**	
			EPEC	***S.* Enteritidis**
CCMA 1284	95.33 ±3.59^{a}	22.09 ±4.23^{b}	2.39 ±3.49^{c}	8.08 ±3.03^{bc}
CCMA 0743	96.06 ±0.26^{a}	38.62 ±2.56^{ab}	13.01 ±6.99^{c}	24.04 ±0.96^{a}
CCMA 0359	80.02 ±1.98^{a}	20.10 ±4.03^{b}	4.57 ±1.20^{c}	13.29 ±2.18^{ab}
CCMA 0504	46.49 ±4.53^{b}	45.36 ±6.30^{ab}	65.15 ±1.52^{a}	NC^{c}
CCMA 0505	35.29 ±3.11^{b}	52.66 ±4.98^{a}	38.10 ±4.76^{b}	9.76 ±2.44^{bc}

Note: Mean of 3 experiments, each was carried out in triplicate ± Standard Error.
Different letters for values at the same column mean significant different values according to the Tukey test at a 95% confidence level. NC means that no co-aggregation ability was found.

Phenol Tolerance

The effect of the two different phenol concentrations (0.2% and 0.5%) [39, 40] evaluated on the growth of the five selected LAB is shown in Fig. 1. There were differences in the sensitivities of the strains for the different evaluated phenol concentrations. As expected, the strains were more tolerant to 0.2 % phenol than 0.5%. In 0.2% phenol, *L. paracasei* CCMA 0504 was the most tolerant (58.93%), followed by *L. plantarum* CCMA 0743 (53.11%). In 0.5%

phenol, all the evaluated strains had less than 5% relative growth. Divisekera et al. [41] evaluated three *Lactobacillus* spp. and reported that they were not able to tolerate 0.5% phenol.

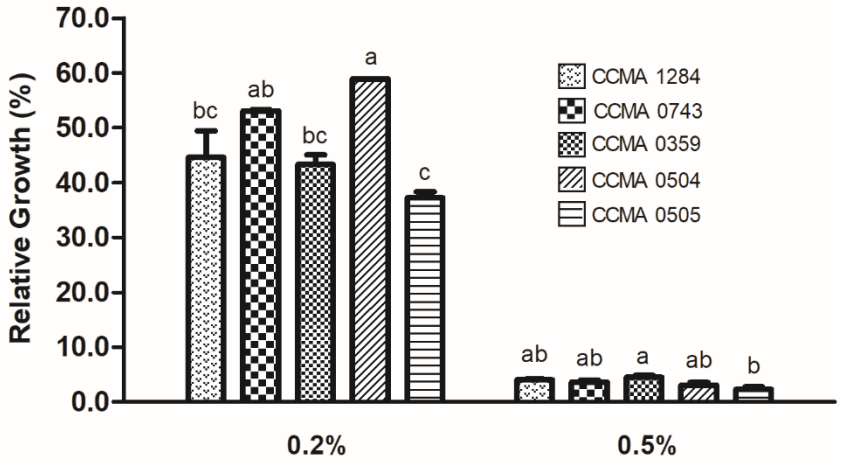

Fig. 1 Effect of phenol concentration on the growth of the five selected LAB strains. Bars indicate standard error of the mean of three independent experiments. The different superscript letters at the same phenol concentration indicate significant differences ($p < 0.05$) by the Tukey test

Phenols are compounds formed after bacterial degradation of aromatic amino acids and have been shown to exert toxic effects. Their presence can be affected by many factors such as diet, endogenous proteins, and gut microbiota composition [42, 43]. Phenols inhibit various species of bacteria and may, therefore, affect the diversity and metabolic activity of the intestinal microbiota, mainly by the formation of more potent inhibitory compounds, such as phenolic acids, resulting from microbial transformations of flavonols, flavan-3-ols, flavones, and anthocyanins obtained from the diet [44]. Furthermore, most polyphenols follow through the colon, where maybe converted by many intestinal bacteria in short-chain fatty acids that can modulate intestinal microbiota composition, increasing *Lactobacillus* genus e other beneficial bacteria populations [45]. Taken together, these results suggest that phenolic compounds resulting from deamination of aromatic amino acids by the intestinal microbiota have bacteriostatic effects against some probiotic strains [9]. Thus, phenol tolerance is an interesting issue for probiotic strains characterization.

Antimicrobial Activity

Regarding antimicrobial activity, *L. plantarum* CCMA 0743 and *L. paracasei* (CCMA 0504 and CCMA 0505) demonstrated inhibitory activity against all pathogens evaluated (Table 5). The two *L. paracasei* strains and *L. plantarum* CCMA 0743 showed the highest activity against *S. aureus* and *B. cereus*, respectively. Conversely, *L. brevis* CCMA 1284 and *L. plantarum* CCMA 0359 showed no inhibitory activity for the evaluated pathogenic microorganisms. *Lactobacillus* spp. have been identified with different antibacterial activities against a range of human pathogens [46].

Table 5 Inhibitory activity of selected LAB strains

Strains	**Indicator strains**				
	SE	**EC**	**BC**	**LM**	**SA**
CCMA 1284	-	-	-	-	-

CCMA 0743	++	++	+++	++	++
CCMA 0359	-	-	-	-	-
CCMA 0504	+	+	++	++	+++
CCMA 0505	+	+	++	++	+++

-: no inhibition, +: inhibition zone 9-11 mm, ++: inhibition zone 12–14 mm, +++: inhibition zone ≥ 15 mm.
SE: *S.* Enteritidis ATCC 564; EC: *E. coli* CDC 055; BC: *B. cereus* ATCC 14579; LM: *L. monocytogenes* ATCC 19117; SA: *S. aureus* ATCC 5674.

Antibiotic Susceptibility

The evaluated strains were sensitive to at least one antibiotic from the cell wall synthesis inhibitor and protein synthesis inhibitor classes. All strains were sensitive to ampicillin and chloramphenicol, resistant to vancomycin, streptomycin, and gentamicin, and showed some sensitivity to erythromycin (Table 6). Only the two *L. paracasei* strains were moderately susceptible or susceptible to lincomycin, azithromycin, and penicillin, while the *L. brevis* CCMA 1284 strain was resistant to these three antibiotics. Several *Lactobacillus* spp. have been reported to be vancomycin-resistant [26, 31], which was also observed in the present study. Antibiotic resistance may become a risk if associated with gene transfer [47]. However, in most cases it is not a cause for concern as it maybe not of the transmissible type, nor it is a specific characteristic of the microbial genus or species [48]. Therefore, these resistance mechanisms may be intrinsic to the strain as demonstrated by Handwerger et al. [49], who reported on vancomycin-resistant *Lactobacillus* spp. These authors suggested that the antibiotic-resistant strains, which are not associated with gene transfer, are interesting candidates for concomitant therapy or after antibiotic use, thereby decreasing the adverse effects of these drugs. However, this parameter was not evaluated in the present study and should be considered for further characterization of selected candidates. Finally, all strains showed some degree of susceptibility to novobiocin (a gyrase inhibitor), while only the two *L. paracasei* strains were susceptible to 1 µg oxacillin (an inhibitor of cell wall synthesis); both antibiotics had no proposed scores.

Table 6 Antibiotic susceptibility[a] of selected LAB strains evaluated by disc diffusion method

Strains	**Diameter of inhibition zone in mm of the antibiotic tested[b]**										
	VAN30	**AMP10**	**PEN10**	**S10**	**GEN10**	**CLO30**	**E15**	**AZI15[c]**	**L2[d]**	**OXA1[e]**	**NV30[e]**
CMA 1284	0 (R)	22 (S)	14 (R)	0 (R)	11 (R)	26 (S)	18 (S)	13 (R)	0 (R)	0	12
CCMA 0743	0 (R)	20 (S)	20 (MS)	0 (R)	0 (R)	24 (S)	16 (MS)	12 (R)	0 (R)	0	15
CCMA 0359	0 (R)	28 (S)	18 (R)	0 (R)	11 (R)	28 (S)	18 (S)	14 (MS)	0 (R)	0	16
CCMA 0504	0 (R)	24 (S)	28 (S)	7 (R)	0 (R)	24 (S)	22 (S)	20 (S)	10 (MS)	10	11
CCMA 0505	0 (R)	24 (S)	26 (MS)	0 (R)	0 (R)	18 (S)	26 (S)	21 (S)	10 (MS)	10	14

[a]The susceptibility of the isolates was scored as resistant (R), moderately susceptible (MS) and susceptible (S) according to the cut-off values proposed by Charteris et al. (1998).
[b]Antibiotics: VAN = vancomycin (30 µg); AMP = ampicillin (10 µg); PEN = penicillin (10 µg); S = streptomycin (10 µg); GEN = gentamicin (10 µg); CLO = chloramphenicol (30 µg); E = erythromycin (15 µg); AZI = azithromycin (15 µg); L = lincomycin (2 µg); OXA = oxacilina (1 µg); NV = novobiocin (30 µg).
[c]Reference to macrolides group.
[d]Reference to lincosamides group.
[e]No values proposed.

In vitro Assessment of Safety Attributes

The determination of safety characteristics is one of the criteria for selecting novel probiotic strains [50]. All five isolates did not show hemolytic and gelatinase activities when compared to the positive control strain, *S. aureus* ATCC 25923. Regarding DNAse activity, *L. paracasei* CCMA 0504 and CCMA 0505 strains were positive. Previous studies have reported the presence of extracellular DNAse in *L. plantarum* [51] and that the secretion of this enzyme may be found in milk-related *Lactobacillus* [52]. In the present study, the positive DNAse strains were isolated from kefir. Although the production of these enzymes has been considered as a virulence factor [53], nucleases secreted by *Lactobacillus* spp. have demonstrated activity against Gram-negative bacteria and bacteriophages, and may be associated with nutritional functions [52].

Adhesion of LAB Strains to Caco-2 and HT-29 Cell Lines

The adhesion capacities of beneficial bacteria and pathogens may be affected by the *in vitro* cell line used for evaluation as well as the mechanisms of the strain interacting with superficial components of intestinal cells; this is mainly related to the production (or not) of mucus [13]. The present study evaluated LAB adhesion to two different cell lines, Caco-2 and HT-29. The percentage adhesion of the strains did not differ ($p>0.05$) from the positive control strain *L. paracasei* LBC-81 on HT-29 cells. On the other hand, *L. paracasei* CCMA 0505 showed higher ($p<0.001$) adhesion capacity (4.75%) than the positive control strain (0.85%) on Caco-2 cells (Fig. 2). Adhesion capacity is influenced by cell surface components and by specific adhesive proteins expressed on this surface that can confer varying degrees of adhesive properties [54].

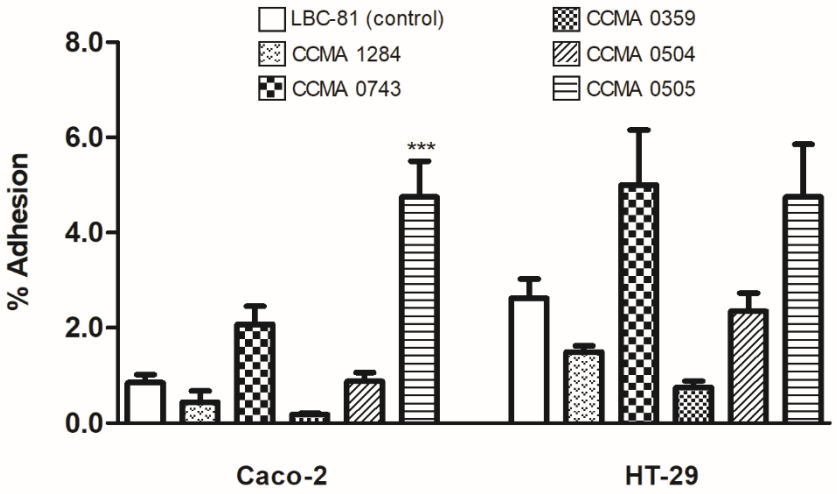

Fig. 2 Adhesion capacity of LAB strains to Caco-2 and HT-29 cells. The adhesion capacity is calculated using the ratio of the number of bacterial cells that remained attached to the total number of bacterial cells initially added to each well. Asterisks indicate statistically significant differences: *p<0.05, **p<0.01, ***p<0.001 compared to the control, using Dunnett's test. The results are expressed as the mean ± SEM of three independent assays

Inhibition of pathogenic bacteria adhesion to Caco-2 and HT-29 cells

The inhibition of *Escherichia coli* (EPEC) CDC 055 and *Salmonella enterica* serovar Enteritidis ATCC 564 adhesion by the five selected LAB strains was evaluated by competition and exclusion assays. A significant reduction (0.7 to 1.7 Log CFU/mL) in *E. coli* adhesion to Caco-2 cells by the competition test was observed in the presence of all evaluated strains except CCMA 1284, while in the exclusion test, CCMA 1284 and CCMA 0743

were not able to reduce pathogen counts ($p \geq 0.05$) (Fig. 3A). On the other hand, for the assays using HT-29 cells, only CCMA 0743 and CCMA 0505 were able to significantly ($p < 0.01$) reduce *E. coli* adhesion (Fig. 3B).

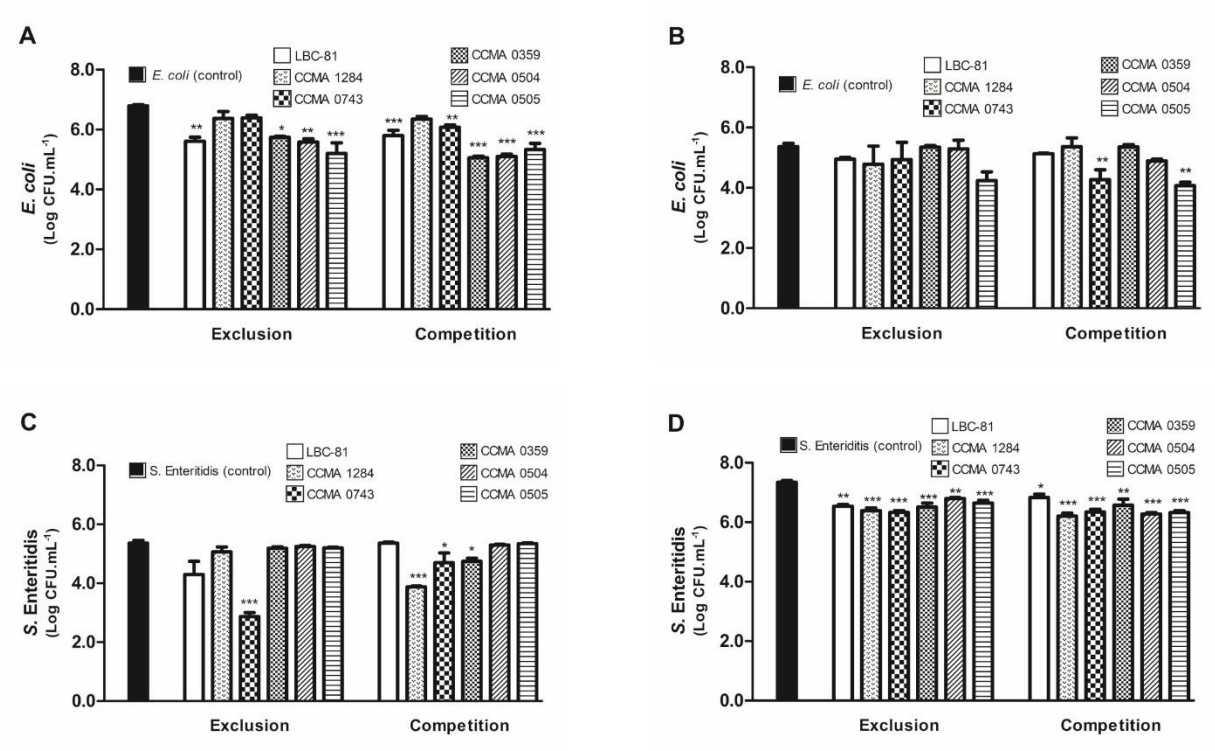

Fig. 3 Effect of *Lactobacillus* strains on the adhesion of enteropathogens to intestinal cell lines. Caco-2 (A and C) and HT-29 (B and D) cells were incubated with *E. coli* and *S.* Enteritidis alone, or in the presence of *Lactobacillus* strains. Asterisks indicate significant differences: *p<0.05, **p<0.01, ***p<0.001, compared to the control, according to Dunnett's test. The results are expressed as the mean ± SEM of three independent assays

The adhesion of *S.* Enteritidis to Caco-2 cells was significantly ($p < 0.05$) reduced by competition with CCMA 1284, CCMA 0743 and CCMA 0359 strains, but in the exclusion assay only the CCMA 0743 strain reduced ($p < 0.001$) pathogen counts (approximately 2.5 Log CFU/mL) (Fig. 3C). All the five evaluated LAB strains were able to inhibit ($p < 0.05$) the adhesion of *S.* Enteritidis to HT-29 cells in both exclusion and competition assays (Fig. 3D).

Studies have reported that *Lactobacillus* strains may inhibit pathogen adhesion by preventing their colonization through competitive exclusion, a highly specific mechanism that is strain-dependent for both probiotics and pathogens [18]. However, adhesion inhibition may be related to different mechanisms such as antimicrobial substances produced by LAB, competition for eukaryotic cell receptors and substrates, the intestinal-mucosal barrier, immunomodulation, and co-aggregation [33]. In the present study, there was a reduction of approximately 1 Log CFU/mL of *S.* Enteritidis to HT-29 cells in the presence of *L. plantarum* CCMA 0743 by the exclusion assay.

According to Gagnon et al. [13], *Salmonella* has higher adhesion and invasion capacities in mucus-producing intestinal cell models (HT-29-MTX) than in non-mucus-producing cells (Caco-2). The adhesion of *S.* Enteritidis to the Caco-2 and HT-29 cells observed in this study corroborate the results of these authors, as we observed adherent bacteria counts of 5.4 Log CFU/mL and 7.3 Log CFU/mL in non-mucus producing (Caco-2) and low mucus-producing (HT-29) models, respectively (Fig. 4C and D). In contrast, EPEC adhered more strongly to Caco-2 cells (6.8 Log CFU/mL) than HT-29 cells (Fig. 4A). There is evidence that the ability of pathogenic

bacteria to colonize and invade cells of different mucosal surfaces is directly related to the expression of specific proteins, pili, fimbriae and flagella [55].

Conclusion

Of the 19 strains that were able to tolerate and survive in simulated gastric and intestinal juices, only five expressed remarkable cell surface characteristics (hydrophobicity and auto-aggregation). These strains were able to reduce the colonization and invasion of EPEC and *S.* Enteritidis in human epithelial cells (Caco-2 and HT-29), with *L. plantarum* CCMA 0743, *L. paracasei* CCMA 0504, and *L. paracasei* CCMA 0505 also exhibiting antimicrobial activity towards pathogenic bacteria. However, special attention should be given to *L. paracasei* strains due to their ability to secrete DNAse, whose properties and mechanisms of action must be elucidated.

Among these strains, *L. plantarum* CCMA 0743, which is isolated from cauim, an indigenous fermented beverage for infants, exhibited interesting probiotic properties, making it the most promising candidate. However, *in vivo* evaluation of these probiotic effects is required to confirm these findings. Moreover, the analysis of the potential of this probiotic strain for biotechnological development is still needed before its therapeutic applications can be defined.

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ARTIGO 2

Versão preliminar nas Normas do periódico *Food and Bioprocess Technology* (ISSN: 1935-5149)

Lactiplantibacillus plantarum CCMA 0743 and *Lacticaseibacillus paracasei* subsp. *paracasei* LBC-81 metabolism during the single and mixed fermentation of tropical fruit juices

Abstract

Fruit juices have shown promising results as new probiotic carriers. Phytochemical modification of three different juices fermented by lactic acid bacteria at 37 °C/24 h were evaluated. Acerola, jelly palm, and passion fruit juices were used as substrates for fermentation of single or mixed *Lactiplantibacillus plantarum* CCMA 0743 and *Lacticaseibacillus paracasei* LBC-81 cultures. After 18 h of fermentation, passion fruit juice showed higher viability of single or mixed *L. plantarum* CCMA 0743 culture, above 9.00 Log CFU/mL, and pH between 4.07-4.10. Sugars consumption and organic acid production were influenced by substrate and culture used. The mixed culture reduced the total of sugars in the passion fruit juice by approximately 53.0% (8.51 g/L). Lactic acid was the main product of the sugars fermentation, with higher concentrations detected in passion fruit juice (8.39-11.23 g/L). Bioactive compounds were analyzed on the selected substrate. The fermentative process reduced antioxidant activity and carotenoid content. However, single *L. plantarum* CCMA 0743 culture increased yellow flavonoid content in passion fruit juice by approximately 3.0 µg/mL. Thus, *L. plantarum* CCMA 0743 showed high cell viability and increased bioactive compounds in passion fruit juice.

Keywords

Functional food. Health benefits. LAB. Nutrient. Probiotic. Vegetable.

Introduction

Fermentation is an ancient method of food preservation, which also improves the nutritional properties of foods. Fermented beverages have become known for their functional attributes in many regions of the world. Additionally, non-dairy substrates, including fruit juices, have been making efforts to innovate and develop new fermented beverages (Marsh et al. 2014; Pimentel et al. 2021).

The fermentation modifies the foods' composition by microbial metabolism and can improve several health benefits (Septembre-Malaterre et al. 2018). For examples, lactic acid bacteria (LAB), especially *Lactiplantibacillus*

plantarum (former *Lactobacillus plantarum*) specie, have been related to increasing bioactive compounds, such as anthocyanin, phenolic, flavonoid, and antioxidant activity, of mulberry (Kwaw et al. 2018), *Momordica charantia* (Gao et al. 2019), and oranges juices (Multari et al. 2020). Another successful observation has been documented by indigenous *L. plantarum* CCMA 0743 strain (Freire et al. 2017). This strain showed interesting *in vitro* probiotic properties, including reducing pathogen colonization in human epithelial cells (Fonseca et al. 2020).

Nowadays, fruit juices have been reported as appropriate substrates for lactobacilli cultures. Studies have reported that commercial and non-commercial LAB species may show high cell viability in vegetable fermentation (Cirlini et al. 2020; Hashemi and Jafarpour 2020; Ricci et al. 2018). On the other hand, some researches have observed cell viability reduction after fruit juice fermentation (Kaprasob et al. 2017; Roberts et al. 2018). Although juices contain essential nutrients (minerals, vitamins, dietary fibers, antioxidants), some parameters such as acidity, antinutrients presence, processing, and others can limit LAB survival in juices (Tripathi and Giri 2014). Thereby, strain and substrate choices become essential criteria for fermentation success, since the microbial metabolism is entirely dependent on the interaction of these intrinsic parameters and extrinsic parameters. Fermented foods are significantly influenced by the single or mixed fermentation of LAB or fruit-based substrate (Espirito-Santo et al. 2015; Zhao et al. 2019).

Tropical fruits, both cultivable and native, have increased recognition of their nutritional and functional values. Researches have demonstrated that passion fruit, acerola, and jelly palm fruits show interesting nutritional properties as relevant sources of bioactive compounds (Da Silva et al. 2014; Magalhaes et al. 2017; Rufino et al. 2010). Among these exciting properties, acerola display a considerable amount of vitamin C (Rufino et al. 2010), jelly palm high antioxidant capacities (Pereira et al., 2013), and passion fruit great total phenolic content (Ramaiya et al. 2013).

Based on the fact that the fermentation process is dependent on the substrate and the starter culture, this work aimed to evaluate acerola, jelly palm, and passion fruit juices as substrates for fermentation using two LAB strains in single and mixed cultures. First, the juices were evaluated as substrate and selected based on bacterial growth performance during fermentation. Afterward, the impact of fermentation on sugars, organic acids, and bioactive compounds were also appraised.

Materials and Methods

Fruit juices preparation

The passion fruit (*Passiflora edulis*), acerola (*Malpighia emarginata*), and jelly palm (*Butia capitata*) pulps were obtained from Cooperativa Grande Sertão (Montes Claros, Minas Gerais - Brazil). The juices were prepared by diluting

frozen pulp in potable water (1:5) as recommended by the manufacturer. After dilution, the juices' pH was adjusted to 5.6 with sterile 3M NaOH, pasteurized at 80 °C for 5 min, and then cooled at 37 °C.

Microorganisms and inoculum preparation

The strains *Lactiplantibacillus plantarum* CCMA 0743 (from the Culture Collection of Agricultural Microbiology) and *Lacticaseibacillus paracasei* subsp. *paracasei* LBC-81 (Danisco, USA) were used in this study. The *L. plantarum* CCMA 0743 strain was used due to its probiotic potential evaluated previously (Fonseca et al. 2020). Inoculums were prepared by transferring the stock culture of *L. plantarum* CCMA 0743 and *L. paracasei* LBC-81 to MRS broth. Strains were twice subcultured statically at 37 °C to obtain an active cell population culture of 8.0 Log CFU/mL, which were then used as starter cultures in the juice fermentations.

Fermentations

Fermentations were performed in Erlenmeyer flasks (250 mL) containing 150 mL of prepared juices and inoculated with 1% (v/v) (~ 6.0 Log CFU/mL) of CCMA 0743, LBC-81, and CCMA 0743 + LBC-81 (1:1). The samples were statically fermented for 24 h at 37 °C. Viable cells and pH were evaluated at every 6 hours of fermentation. The fruit juice and the fermentation time, which showed lower pH and higher viable cell counts, were selected for further analysis.

Microbiological analysis and pH determination

Microbiological analysis, growth media, and incubation conditions were performed according to Szutowska et al. (2020), with modifications. Serial dilutions of fermented juices were performed using sterile peptone water (0.1% w/v), and aliquots of appropriate dilutions were plated in duplicated by spread plate method. Viable cell counts of the lactic acid bacteria strains were enumerated on MRS (Kasvi, Italy) agar at 37 °C for 48 h under aerobic conditions. Results were expressed as Log CFU/mL.

The juices pH was determined by direct measurement in a Digimed DM-22 potentiometer.

Sugars and organic acids determination

Organic acids (lactic, citric, malic, succinic, acetic, and butyric acid) and sugars (glucose, fructose, and sucrose) analysis were performed as described by Freire et al. (2017), with modifications. A Shimadzu Liquid Chromatography System (Shimadzu Corp., Japan), equipped with a dual detection system consisting of a UV–Vis detector (SPD 10Ai) (for acids) and a refractive index detector (RID-10Ai) (for sugars) was used. Organic acids were determined using a Shimadzu Ion Exclusion Column, Shim-pack SCR-101H (300 mm × 7.9 mm i.d., 10 μm) at an operating temperature of 50 °C, using an aqueous solution of perchloric acid as mobile phase. Sugars were analyzed using a Shimadzu ion exclusion column, Shim-pack SCR-101C (300 mm × 7.9 mm i.d., 10 μm) at an operating temperature of 80 °C, using water as mobile phase. The acids and sugars were identified by comparison with retention times of authentic standards. The quantification was performed using calibration curves constructed with standard compounds. All samples were analyzed in triplicate.

Total phenolic and antioxidant activity determination

The extracts were obtained according to the method described by De Souza et al. (2012), with slight modifications. Briefly, 5 mL of the samples were added in centrifuge tubes and extracted sequentially with 10 mL of methanol/water (50:50, v/v) at room temperature (25 °C) for 1 h. The tubes were centrifuged at 5,240×g at room temperature for 10 min, and the supernatant was recovered. Then, 10 mL of acetone/water (70:30, v/v) was added to the residue at room temperature. The samples were extracted for 1 h and centrifuged under same conditions as before. The methanol and acetone extracts were used for the determination of antioxidant activity and phenolic content as follows.

Total phenolic content determination

Total phenolic content was determined by the Fast Blue (FB) method described by Medina (2011), with some modifications. Sample (2 mL) was homogenized with 0.2 mL of 0.1% (w/v) Fast Blue BB reagent and 0.2 mL 5% (w/v) NaOH for 1 min. The reaction was completed at room temperature for 90 min, and the absorbance was measured spectrophotometrically at 420 nm. A calibration curve was prepared using a gallic acid solution (20-200 μg/mL). Results were expressed as μg of gallic acid equivalent per mL of sample (μg GAE/mL).

Phosphomolybdenum complex method (PCM)

Antioxidant activity was determined by the PCM according to the modified methodology described by Prieto et al. (1999). An aliquot of 0.1 mL of the sample solution was placed in tubes and mixed with 3 mL of reagent solution (1.8 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were capped and

incubated in a water bath at 95 °C for 90 min. Then, the samples were cooled down to room temperature, and the absorbance of the green phosphomolybdenum complex was measured at 695 nm. A mixture containing methanol 50% and acetone 70% (1:1) was used as a blank. The quantification was based on a standard curve of ascorbic acid (1.95 to 500 µg), and the results were expressed in mg ascorbic acid equivalents (AAE) per mL of sample.

ABTS assay

The antioxidant activity was determined using the ABTS (2,20-azinobis-3-ethylbenzothiazoline-6-sulfonate) assay according to the method of Re et al. (1999) with minor modifications. The ABTS radical cation ($ABTS^{+}$) was generated by the reaction of 5 mL of aqueous ABTS solution (7 mM) with 88 µL of 140 mM (2.45 mM final concentration) potassium persulphate. The mixture was kept in the dark for 16 h before use and then diluted with ethanol to obtain an absorbance of 0.7 ± 0.05 units at 734 nm using a spectrophotometer. The juice extracts (30 µL) or a reference substance (Trolox) were allowed to react with 3 mL of the resulting blue-green ABTS radical solution in the dark. The decrease in absorbance at 734 nm was measured after 6 min. The quantification was based on a standard curve of Trolox (0.1 to 2 mM), and the results are expressed as micromoles of Trolox equivalents (TE) per milliliter of the sample (µmol of TE/mL).

Total carotenoids content determination

The extraction of total carotenoids was carried out according to Carbonell-Capella et al. (2015), with slight modifications. Sample (2 mL) was homogenized with 5 mL of extracting solvent (hexane/acetone/ethanol, 50:25:25, v/v) and centrifuged for 5 min at 4,520×g at 4 °C. The top layer of hexane containing the color was recovered and transferred to a 25 mL volumetric flask. The volume of recovered hexane was then adjusted to 25 mL with hexane. The total carotenoid determination was carried out on an aliquot of the hexane extract by measuring the absorbance at 450 nm. Total carotenoids were calculated using an extinction coefficient of β-carotene, $E^{1\%} = 2505$.

Anthocyanins and yellow flavonoids determination

Anthocyanins and yellow flavonoids determination were carried out as described by Francis (1982), with modifications. Briefly, 2.5 mL of each juice sample was suspended in 20 mL of extraction solution (ethanol 95%:1.5 N HCl - 85:15 v/v). Samples were homogenized for 1 min, transferred to a 50 mL volumetric flask, completed the volume with the same extraction solution, and extracted for 16 h under refrigeration (7 °C) in the dark. After this period, the

extracts were filtered, and the absorbances at 535 nm (anthocyanins) and 374 nm (yellow flavonoids) were measured. The content of anthocyanins and yellow flavonoids were calculated using equation 1 and absorption coefficients of 98.2 and 76.6 (mol/cm), respectively.

$$\text{Yellow flavonoids content (μg/mL)} = \frac{(\text{ABS} \times \text{dilution factors}) \times 104}{(\text{sample volume} \times \varepsilon^{1\%}_{1\text{ cm},374})} \quad (1)$$

where ABS is the absorbance reading of the sample at 374 nm and $\varepsilon^{1\%}_{1\text{ cm},374}$ is the absorption coefficient for yellow flavonoids. Anthocyanins content was calculated using the same equation with absorbance reading at 535 nm and its respective absorption coefficient.

Determination of ascorbic acid content

The ascorbic acid content was determined using the iodine titration method modified from Suntornsuk et al. (2002). One milliliter of juice was transferred into a 125 mL conical flask and diluted with 4 mL distilled water. Five milliliters of 2 N sulfuric acid was added, mixed, and 2 mL of 1% starch was added as an indicator. The solution was directly titrated with a 0.001 N standardized iodine solution. A blank titration was performed before the titration of each sample. Each milliliter of 0.001 N iodine was equivalent to 88.06 μg of ascorbic acid.

Statistical analysis

All the treatments and assays were carried out thrice, and results were presented as mean ± standard error. The analysis of variance (ANOVA) was performed, and the Tukey test was used to calculate significant differences at $p < 0.05$. Principal Component Analysis (PCA) was applied to the data set through multivariate exploratory techniques. It was chosen to include just enough *k* components to explain at least 80% of the total variance. The analyzes were performed using Statistica software version 10.0 (Statsoft, USA).

Results

***Lactobacillus* growth and acidification**

In general, *L. plantarum* and *L. paracasei* strains, in the single or mixed assays, showed high viability during fermentation in the three evaluated fruit juices (Fig. 1). However, in single or mixed cultures, the potentially probiotic strain CCMA 0743 showed faster adaptation to the juices, as demonstrated by increasing around 2 Log CFU/mL at 6 h of fermentation. At the same time, the commercial probiotic strain in single culture reached the desired counts above 8 Log CFU/mL only in the jelly palm juice. After 12 h of the fermentative process in all evaluated juices, no significant increase in cell counts was observed for all cultures. The highest cell number (8.95 – 9.11 Log CFU/mL) was observed for *L. plantarum* CCMA 0743, in single or mixed cultures, in the passion fruit juice from 12 h of fermentation.

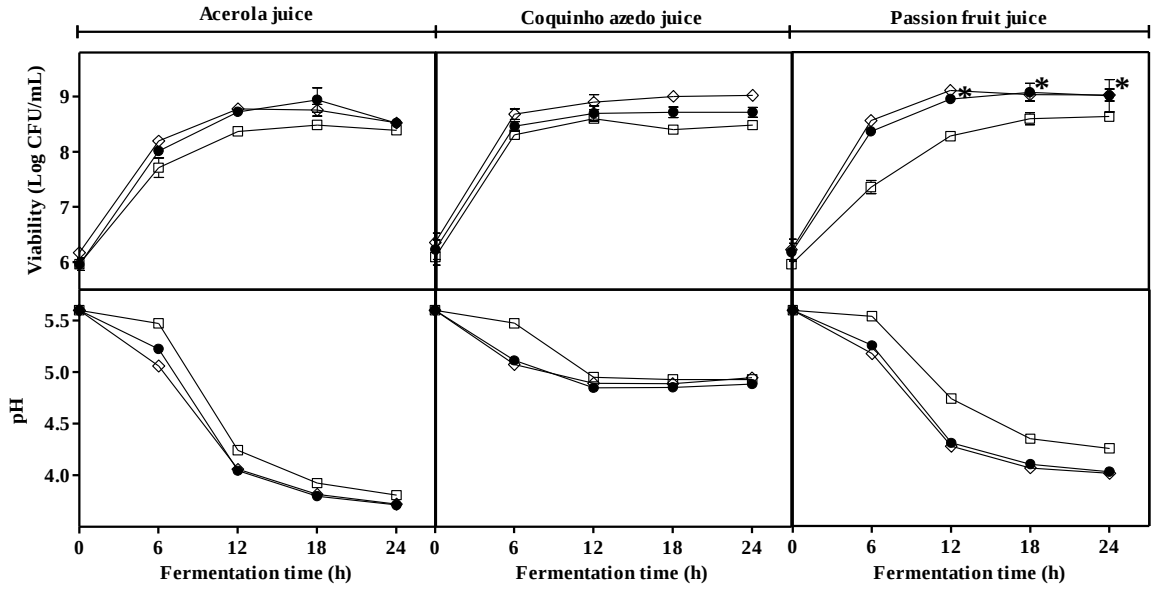

Fig. 1 Variations in growth and pH during 24 h of fermentation at 37 °C of acerola, jelly palm, and passion fruit juices fermented by LAB strains. Asterisks indicate greater cell viability, regardless of the substrate used and fermentation time. Bars represent standard error of triplicate measurements of three independent assays. *L. plantarum* CCMA 0743 monoculture (◊); *L. paracasei* LBC-81 monoculture (□); *L. plantarum* CCMA 0743 and *L. paracasei* LBC-81 binary inoculation (●)

A decrease in pH was observed in all juices and assays. The most significant reductions occurred between 6 and 12 h of fermentation. By comparing the juices, the pH reduction in the jelly palm juice fermentation reached values close to 4.9, while in the other two evaluated juices, the pH values were below 4.0 for all strains. Comparing the strains, the single and mixed *L. plantarum* strain showed a higher pH decrease than *L. paracasei* strains in all evaluated juices.

According to the obtained results (Fig. 1), the fermentation time of 18 h was able to maintain an adequate viable cell concentration for the probiotic purpose (above 9 Log CFU/mL), and the pH was reduced to a value around 4.0, which is considered enough to avoid spoilage and to ensure microbiological stability. After 18 h of fermentation, the microbial viability was not significantly improved ($p > 0.05$). Thus, this fermentation time was considered for chemical evaluations.

Sugars and organic acids metabolism

The sugar composition of the fruit juices is shown in Fig. 2A. The passion fruit and jelly palm juices contained the highest (16.13 g/L) and the lowest (1.49 g/L) amount of fermentable sugars. Acerola juice showed 6.48 g/L of total sugars. Passion fruit juice showed higher sucrose concentrations (11.50 g/L), while acerola and jelly palm juices, higher monosaccharide levels.

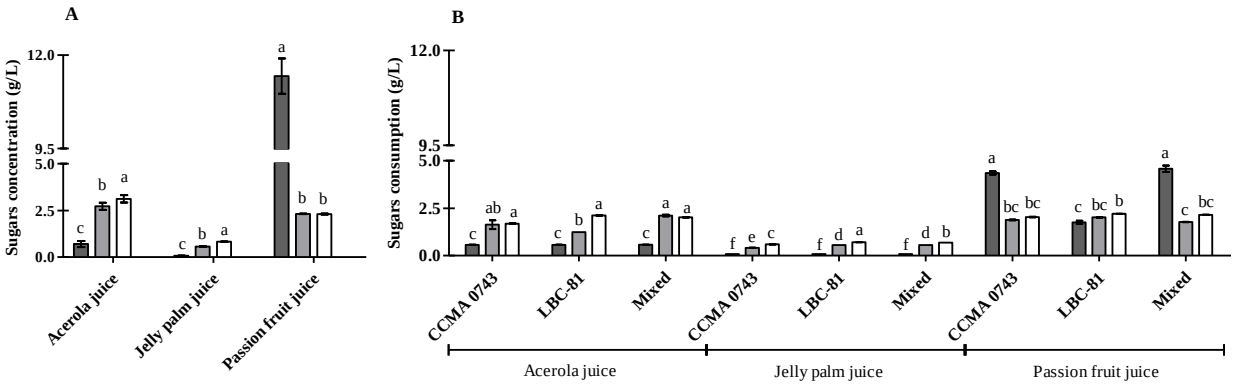

Fig. 2 Sugars content (g/L) of unfermented juices (A) and sugars consumption (g/L) in fermented juices (B). Bars indicate standard error of sugar content of unfermented juices and sugars consumption in juices fermented with single and mixed cultures of *L. plantarum* CCMA 0743 and *L. paracasei* LBC-81 after 18 h of fermentation. The same lowercase letters are not significantly different between treatments within the same study juice at $p>0.05$. sucrose (■); glucose (■); fructose (□)

Fig. 2B shows sugar consumption by the strains in the three evaluated juices. As observed, in acerola and jelly palm juices, the strains exhibited higher glucose and fructose consumption than sucrose. Although there was a lower sucrose consumption than monosaccharides in jelly palm juice, this sugar was not detected after 18 h of fermentation. In contrast, in the passion fruit juice, the strain *L. plantarum* CCMA 0743 in single and mixed showed higher ($p<0.05$) sucrose consumption than the monosaccharides (Fig. 2B). *L. plantarum* CCMA 0743 single or mixed consumed approximately 4.5 g/L of sucrose in the passion fruit juice, a value that corresponds to 39.0% of the total sucrose content. In contrast, glucose and fructose total consumptions in passion fruit juices were approximately 4.0 g/L (86.0% of the total monosaccharides). Regarding passion fruit juice, it seems that sucrose was the preferred sugar. However, the initial concentration of this sugar was higher than the monosaccharides (Fig. 2A). Thus, sugar consumption is affected by the levels of these sugars in the substrate.

The organic acids in the three different juice fermentations were evaluated before and after 18 h of fermentation and are shown in Table 1. Lactic acid was the most abundant organic acid produced after fermentation and was detected

in all fermented juices, ranging from 2.36 to 11.23 g/L. The strain *L. paracasei* LBC-81 in single culture produced lower lactic acid content in acerola juice (5.26 g/L), while the higher production was in jelly palm juice (3.36 g/L). Citric acid decreased significantly ($p<0.05$) after fermentation in jelly palm juice for all evaluated strains, which may be related to the low consumption of fermentable sugars in this juice. On the other hand, malic acid was not detected after fermentation in all juices. Succinic acid decreased in all fermentations, except in acerola juice fermented by *L. paracasei* LBC-81. According to the obtained results, it seems that succinic acid catabolism was higher for the *L. plantarum* CCMA 0743 strain in single or mixed cultivation than for single *L. paracasei*. Acetic acid was produced in jelly palm juice fermentation and acerola juice fermented by *L. paracasei* LBC-81 in the single culture, with concentrations ranging from 0.23 to 1.12 g/L. It may suggest that the acetic acid was a result of the citric acid fermentation. Butyric acid was not detected in the acerola juice at any evaluated time of the fermentation process. However, this organic acid was consumed in passion fruit juice, while in jelly palm juice, this acid was produced by the three evaluated starter cultures.

Table 1 Changes in organic acid of the fermented juices by lactic acid bacteria cultures

Juice	Fermentation time	Strain	Organic acid (g/L)					
			lactic acid	citric acid	malic acid	succinic acid	acetic acid	butyric acid
Acerola	Before fermentation (0 h)		ND^{c}	0.17 ±0.01^{a}	0.66 ±0.03^{a}	0.46 ±0.03^{a}	ND^{b}	ND^{a}
	After fermentation (18 h)	0743	7.49 ±0.19^{a}	0.15 ±0.03^{a}	ND^{b}	0.03 ±0.01^{b}	ND^{b}	ND^{a}
		81	5.26 ±0.46^{b}	ND^{a}	ND^{b}	0.37 ±0.03^{a}	0.23 ±0.03^{a}	ND^{a}
		Mix	7.76 ±0.13^{a}	0.13 ±0.01^{a}	ND^{b}	0.03 ±0.01^{b}	ND^{b}	ND^{a}
Jelly palm	Before fermentation (0 h)		ND^{c}	2.99 ±0.11^{a}	2.16 ±0.03^{a}	6.30 ±0.02^{a}	ND^{d}	ND^{b}
	After fermentation (18 h)	0743	2.72 ±0.17^{b}	1.26 ±0.17^{b}	ND^{b}	0.78 ±0.01^{c}	0.35 ±0.01^{c}	0.27 ±0.01^{a}
		81	3.36 ±0.03^{a}	0.71 ±0.01^{bc}	ND^{b}	3.97 ±0.02^{b}	1.12 ±0.01^{a}	0.26 ±0.01^{a}
		Mix	2.36 ±0.02^{b}	0.57 ±0.04^{c}	ND^{b}	0.85 ±0.03^{c}	0.94 ±0.01^{b}	0.27 ±0.01^{a}
Passion fruit	Before fermentation (0 h)		0.06 ±0.02^{b}	33.60 ±0.88^{a}	0.60 ±0.02^{a}	0.27 ±0.01^{a}	ND^{a}	0.45 ±0.06^{a}
	After fermentation (18 h)	0743	9.67 ±1.32^{a}	27.73 ±3.88^{a}	ND^{b}	ND^{b}	ND^{a}	ND^{b}
		81	8.39 ±0.26^{a}	32.98 ±0.52^{a}	ND^{b}	ND^{b}	ND^{a}	ND^{b}
		Mix	11.23 ±0.06^{a}	30.99 ±0.16^{a}	ND^{b}	ND^{b}	ND^{a}	ND^{b}

Means ± standard error in the same column for each juice, followed by different lowercase letters, indicate statistically significant differences at $p \leq 0.05$, according to Tukey test (n = 3). 0743 = *L. plantarum* CCMA 0743 monoculture; 81 = *L. paracasei* LBC-81 monoculture; Mix = *L. plantarum* CCMA 0743 and *L. paracasei* LBC-81 binary inoculation. ND = Not detected.

Based on the high cell viability, fast pH reduction, sugars consumption, and organic acid production, the passion fruit juice fermented by CCMA 0743 strain were selected for further analysis.

Bioactive compounds

Antioxidant activity and bioactive compounds were evaluated and are shown in Table 2. As observed, the antioxidant activity and carotenoid levels of passion fruit juice were reduced ($p<0.05$) after fermentation. The highest antioxidant activity decrease (around 40%) was obtained by the mixed culture evaluated by ABTS and PCM methods (Table 2). A reduction ($p<0.05$) of about 30% in carotenoid concentrations (3.7 to 2.6 µg/mL) was also observed for single and mixed cultures. On the other hand, the passion fruit juice fermented by *L. plantarum* CCMA 0743 strain increased yellow flavonoid content (74.15 µg/mL versus 71.26 µg/mL), demonstrates that LAB activity can enhance this phytochemical concentration in passion fruit juice. There was no difference ($p>0.05$) in total phenolic and anthocyanin contents of the evaluated unfermented and fermented juices. Furthermore, even after the pasteurization and fermentation processes, there was no significant modification ($p>0.05$) in the ascorbic acid levels, which showed 67.51 µg/mL and 74.85 µg/mL for single and mixed cultures, respectively.

Table 2 The antioxidant activity (ABTS and PCM), total phenolic, anthocyanin, yellow flavonoid, carotenoid, and ascorbic acid contents of unfermented and fermented passion fruit juices

Treatment	Antioxidant activity		Total phenolic (µg of GAE/mL)	Anthocyanin (µg/mL)	Yellow flavonoid (µg/mL)	Carotenoid (µg/mL)	Ascorbic acid (µg/mL)
	ABTS (µmol of TE/mL)	PCM (mg of AAE/mL)					
Unfermented juice (Control)	8.99 ±0.68^{a}	25.78 ±0.84^{a}	662.91 ±24.70^{a}	1.98 ±0.24^{a}	71.26 ±0.95^{b}	3.74 ±0.02^{a}	86.59 ±3.88^{a}
0743	6.20 ±0.67^{ab}	19.76 ±0.24^{b}	720.48 ±16.25^{a}	1.25 ±0.32^{a}	74.15 ±0.54^{a}	2.61 ±0.10^{b}	67.51 ±1.47^{a}
Mix	5.10 ±0.61^{b}	15.67 ±0.18^{c}	620.89 ±35.66^{a}	1.25 ±0.38^{a}	71.61 ±0.14^{ab}	2.63 ±0.10^{b}	74.85 ±6.73^{a}

Means ± standard error in the same column followed by different lowercase letters indicate statistically significant differences at $p \leq 0.05$, according to the Tukey test (n = 3).

ABTS - 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonate; PCM - phosphomolybdenum complex method; TE – trolox equivalent; AAE – ascorbic acid equivalent; GAE – gallic acid equivalent. 0743 = *L. plantarum* CCMA 0743 monoculture; Mix = *L. plantarum* CCMA 0743 and *L. paracasei* LBC-81 binary inoculation.

Principal component analysis

The PCA was obtained using sugars, organic acids, and bioactive compounds data of unfermented and fermented passion fruit juices. The first three components had eigenvalues greater than 1, indicating that they should be interpreted (Kaiser Criterion). The first principal component (PC1) explained 70.47% of the variability contained in the original variables, whereas the second (PC2) and third (PC3) principal components explained 12.38 and 7.87%, respectively, counting 90.72% of the total variability (Fig. 3). However, as the total variability of PC1 and PC2 was greater than 80%, PC3 was disregarded.

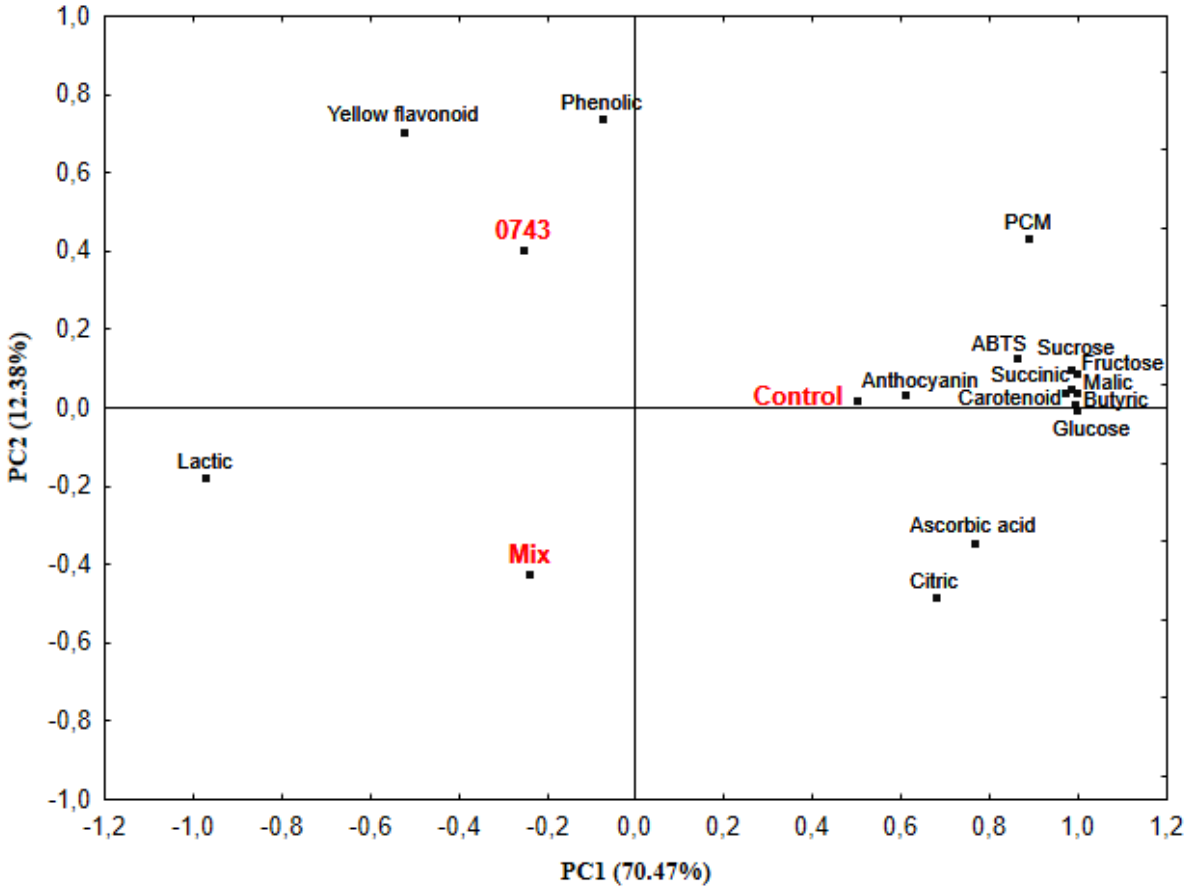

Fig. 3 Principal component analysis of the sugars, organic acids, and bioactive compounds of unfermented and fermented passion fruit juices. Control = unfermented juice; 0743 = passion fruit juice fermented by *L. plantarum* CCMA 0743; Mix = passion fruit juice fermented by *L. plantarum* CCMA 0743 and *L. paracasei* LBC-81; ABTS = 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonate; PCM = phosphomolybdenum complex method

Hence, attributes that showed absolute loading values (>0.700) of each feature were considered significant. In PC1, carotenoid, sugars, succinic, butyric, and malic acids, followed by the antioxidant activity, ascorbic acid, were positively correlated with unfermented treatment, while lactic acid was negatively correlated. This result can be observed

in Fig. 3, where fermented juices were on the negative side, and unfermented juice was on the positive side of PC1. On the other hand, the juice fermented by *L. plantarum* CCMA 0743 was positively correlated with yellow flavonoid and phenolic compounds in the positive side of PC2 and the negative side of PC1.

Discussion

The use of fruit juices as substrates for *Lactobacillus* and emended genera growth have been investigated in recent years (Espirito-Santo et al. 2015; Hashemi et al. 2017; Hashemi and Jafarpour 2020). The choice of the commercial culture *L. paracasei* LBC-81 is because this strain has shown high viability and high production of lactic acid during plant-based beverage fermentation (Menezes et al. 2018). The present study obtained high cell viability (above 8 Log CFU/mL), which is indicated for probiotic purpose, for the three evaluated starter culture in the different fruit juices. However, there were differences in the time of fermentation to reach desired viable cells in the three evaluated fruit juices since they have different profiles of fermentable sugars (Fig. 2A). In addition to the chemical composition of juices, the nutrients availability, the presence and generation of inhibitory compounds, and strains ability to adapt to the stress condition imposed by the fruit juice may be related to the obtained variable fermentation parameters (Espirito-Santo et al. 2015). As a result of fermentation, pH reduction by organic acid production occurs due to the microbial metabolism of carbohydrates (Costa et al. 2013). Thereby, pH value can be used as an indicator of the fermentation progress. The most significant decrease in pH values during fermentation was registered between 6 and 12 h, reaching pH values close to 4.0 in acerola and passion fruit juices. The drop in the pH may be mainly related to lactic acid production that reaches concentration above 5.0 g/L after 18 h of fermentation. This pH result is similar to those previously reported by Costa et al. (2013) evaluating pineapple juice fermented by *Lactobacillus casei* NRRL B-442. In the present study, the highest cell viability and minimal pH values were achieved at 18 h of fermentation in passion fruit juice inoculated with single or mixed CCMA 0743 culture. In addition to fermentative capacity in the juices, this strain has demonstrated probiotic properties (Fonseca et al. 2020), which may be an important option for food industry applications.

The three evaluated starter culture showed the ability to metabolize sucrose, glucose, and fructose. However, the availability of carbohydrates in the different substrates strongly influenced the utilization of carbon source by the microorganism, as reported by Ricci et al. (2019). In parallel, the greater capacity to reduce sucrose levels by the *L. plantarum* CCMA 0743 strain in passion fruit juice (Fig. 1) makes it more advantageous than the commercial culture *L. paracasei* LBC-81 since the low-calorie food product market is in continuous increase. Moreover, this reduction is quite interesting from a nutritional perspective since the excessive consumption of this disaccharide is related to some metabolic diseases (Qi and Tester 2020).

Lactic acid bacteria metabolize carbon sources via fermentation, leading to the formation or degradation of organic acids (Papadimitriou et al. 2016). Among the various LAB, *L. plantarum* and *L. paracasei* belong to the facultatively heterofermentative lactobacilli group (Kõll et al. 2008), producing lactic acid and others as final products. Nevertheless, lactic acid is the primary fermentation final product reported in other studies (Cirlini et al. 2020; Ricci et al. 2019). Malic acid can be converted into lactic acid by malolactic fermentation and is associated with strain adaptation to the acid environment (Ricci et al. 2019). *L. paracasei* and *L. plantarum* species may utilize citric acid to form acetic acid and lactic acid under the catalysis of citrate lyase (Papadimitriou et al. 2016). The increase of acetic acid and citric acid consumption in acerola juice inoculated with *L. paracasei* LBC-81 (Table 1) may be related to citrate lyase action. Normally, lactobacilli metabolize succinate from citrate via the reductive tricarboxylic acid (TCA) pathway. Contrarily, succinic acid was almost totally consumed in most samples (Table 1). Our results corroborate with Lee et al. (2013), and the data likely indicated that acidic conditions are inhibitory to one or more enzyme(s) that would lead to succinate production through the oxidative TCA cycle (Dudley and Steele 2005). Conversion from lactate to butyrate is associated with low energy supply or when bacteria use some prebiotic as source energy (Ningegowda and Gurudutt 2012). However, butyric acid can be used as a precursor to aromatic compounds (Chen et al. 2019). In the present study, strains produced and consumed butyric acid depending on the substrate, as observed, respectively, in jelly palm and passion fruit juices (Table 1). These findings suggest that fruit substrate composition influences microbial metabolism. The higher availability of fermentable sugars associated with some organic acids makes the passion fruit juice an interesting substrate for LAB fermentation.

Bioactive compounds are phytochemicals secondary metabolites synthesized by plants with functional properties and are responsible for the color, flavor, and odor of fruits (Septembre-Malaterre et al. 2018). LAB is recognized by its ability to produce hydrolytic enzymes, increasing phytochemical concentration (Kwaw et al. 2018). However, antioxidant activity and carotenoid content decreased after passion fruit juice fermentation (Table 2). This reduction can probably be attributed to the fermented juice's pasteurization process (process not carried out in unfermented juice) since heat treatment is related to the degradation of antioxidant compounds (Schvab et al. 2015). On the other hand, a production of approximately 3.00 µg/mL of yellow flavonoids by single *L. plantarum* CCMA 0743 culture was observed. It is known that flavonoids possess several important biological activities, such as antioxidant property, anti-inflammatory, antiulcer, antiviral, anti-cancer, anti-diabetic, and cytotoxic (Karak 2019).

Conclusions

The *L. plantarum* CCMA 0743 and *L. paracasei* LBC-81 strains in single and mixed cultures could grow in high concentrations (above 8.0 Log CFU/mL) on all substrates evaluated; however, this population was obtained at different times of fermentation. Furthermore, the different fermentation showed different profiles of sugars and acid metabolisms. The passion fruit showed high sugars source among the juices, and consequently, higher cell viability and low pH value after 18 h of fermentation. Moreover, fermentation using *L. plantarum* CCMA 0743 as the starter culture increased the yellow flavonoid content and consumed exceptional levels of sugars in passion fruit juice. However, it is still necessary to assess the impacts of fermentation on the product's shelf life and changes in volatile aromatic compounds and sensory profiles.

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ARTIGO 3

Versão preliminar nas Normas do periódico *Food Research International* (ISSN: 0963-9969)

Sensory and flavor-aroma profiles of passion fruit juice fermented by potentially probiotic *Lactiplantibacillus plantarum* CCMA 0743 strain

Highlights

The juices maintained suitable LAB counts after simulated digestion.
The juices showed high LAB viability during refrigerated storage.
The fermentation process produced new flavor compounds.
LAB strains differentiate the sensory profile of passion fruit juice.

Abstract

Several non-dairy probiotic beverages are already available to consumers and have been considered suitable carriers for probiotic bacteria. This study aimed to investigate the effect of *Lactiplantibacillus plantarum* CCMA 0743 in single and co-culture on the volatile compounds and sensory profiles of fermented passion fruit juice. The viability of strains inoculated in juice and MRS matrices were evaluated in a simulated gastrointestinal condition. The bacterial viability after 28 days of refrigerated storage of the juices was also evaluated. *L. plantarum* CCMA 0743 showed high viability (6.18 Log CFU/mL) after passage throughout simulated digestion in passion fruit juice matrix. Both juices maintained high probiotic counts (> 8.0 Log CFU/mL) during storage. Also, the yellow color was stable after 28 days of storage. Volatile compounds of passion fruit juices were modified after the fermentation process, such as the degradation of ketones and alcohol formation. The sensory profile of passion fruit juice was modified by single and co-culture fermentations. The fermented samples were mainly correlated with the terminologies "salty, acidic and bitter tastes" and "sweetener aftertaste". Overall, passion fruit juice proved to be an adequate food matrix to deliver the evaluated strains. However, individual strains or strain-strain interactions with the food matrix affect the fermented product, demonstrating that strain and matrices evaluations are essential for developing novel products with acceptable characteristics.

Keywords

Fermentation. Flash profile. Food microbiology. Functional foods. *In vitro* digestion. Lactic acid bacteria. Lactobacilli.

1. Introduction

In recent years, various food matrices have been used to deliver live microorganisms. However, each food matrix has unique properties and advantages but may also impose technological barriers (Min et al., 2019). Therefore, the application of probiotic cultures in plant-based products represents a significant challenge. The suitable choice of food matrix and selection of the probiotic strains are essential to ensure success in manufacturing innovative and attractive healthy food products (Aspri et al., 2020; Vinderola et al., 2017).

Notably, fruit juices have been reported as a novel and appropriate medium for microorganisms, combining nutritional effects with the added value benefits from probiotics (Perricone et al., 2015). Among the desired probiotic characteristics, survival during exposure to the gastrointestinal human tract's harsh adverse conditions is fundamental and may be influenced by food matrices components (De Albuquerque et al., 2018). Moreover, the high viability of probiotic microorganisms during the product's production and storage is crucial to obtain the desired probiotic population and causes health benefits to the host (Tripathi & Giri, 2014). It is well known that fermentation alters the food matrix's composition and can significantly change aromatic compounds and, consequently, sensory attributes (Wei et al., 2018). Thus, evaluating starter culture and food matrices is essential to obtain a probiotic product with high sensory quality and consumer acceptance.

The starter strains generally used for plant origin fermentation products belong to the *Lactiplantibacillus plantarum* (former *Lactobacillus plantarum*) species, recognized to be the most suitably adapted for these substrate types (Garcia et al., 2018; Hashemi & Jafarpour, 2020; Ricci et al., 2019). The effect of lactic acid fermentation on volatile composition and sensory attributes of fruit juices (Alves Filho et al., 2017; Di Cagno et al., 2017), viability post-acidification during refrigerated storage (Chen et al., 2019), and probiotic cells survival in different vegetal matrices (Lopes et al., 2020), demonstrated that *Lactobacillus* spp. and associated genera behave strain-specificity with different food matrix and exhibit different bioconversion capacity (Wu et al., 2020).

Passion fruit (*Passiflora edulis*) is a tropical and subtropical climate and produces fruits for *natura* consumption and juice production (Faleiro et al., 2019). Brazil is the world's leading producer of passion fruit and, in 2019, produced 593,429 tons of fruit (IBGE, 2020). Nutritional properties of passion fruit have been reported in the literature, emphasizing the contents of total carotenoids, total flavonoids, and total polyphenols (Septembre-Malaterre et al., 2016). This fruit is extensively used in traditional medicine worldwide, especially as sedatives and anxiolytics, in addition to utilization by the food industries (Corrêa et al., 2016).

The potential probiotic strain isolated from cauim (*L. plantarum* CCMA 0743) showed interesting *in vitro* probiotic properties, including reducing pathogen colonization in human epithelial cells (Fonseca et al., 2020), besides presenting high viability during single and co-culture fermentations of fruit juices. Based on these results, this study's objective was to assess the passion fruit juice protection of *L. plantarum* CCMA 0743 and *Lacticaseibacillus paracasei* subsp. *paracasei* (former *Lactobacillus paracasei* subsp. *paracasei*) LBC-81 in single and co-culture throughout the gastrointestinal condition compared to the MRS medium. The fermented juices obtained by combining the LAB strains were chemically and microbiologically characterized during the refrigerated storage period. The volatile compounds and sensory profile of the fermented juices were evaluated.

2. Materials and Methods

2.1. Microorganisms and inoculum preparation

The strains *L. plantarum* CCMA 0743 (from the Culture Collection of Agricultural Microbiology, Federal University of Lavras, Brazil) *L. paracasei* LBC-81 (Danisco, USA) were used in this study. The CCMA strain was chosen by its potential probiotic characteristics (Fonseca et al., 2020). Inoculums were prepared by transferring the stock culture of *L. plantarum* CCMA 0743 and *L. paracasei* LBC-81 to 1 mL MRS broth (Kasvi, Italy). Strains were twice subcultured statically at 37 °C to obtain an active cell population culture at 8.0 Log CFU/mL, which was then used to initiate the juice fermentation.

2.2. Fruit juices fermentations and storage

The passion fruit (*Passiflora edulis*) pulp was obtained from Cooperativa Grande Sertão (Montes Claros, Minas Gerais - Brazil). The juices were prepared by diluting frozen pulp in potable water (1:5) recommended by the manufacturer. After dissolution, the juice pH was adjusted to 5.6 with sterile 3 M NaOH, pasteurized at 80 °C for 5 min, and then cooled at 37 °C. The passion fruit juices (300 mL) were bottled in 500 mL screw caps transparent glass bottles and inoculated with 1% (v/v) (~ 6 Log CFU/mL) of single CCMA 0743 culture and co-culture CCMA 0743 + LBC-81 (1:1). The juices were fermented statically for 18 h at 37 °C in a BOD and then stored at 4 °C for 28 days. Samples were obtained at seven days, intervals for pH, cell viability, and color measurements.

2.3. Simulation of gastrointestinal conditions

The simulated gastrointestinal pathway implemented was performed according to De Albuquerque et al. (2018), with slight modifications. Initially, 25-mL aliquots of MRS broth or passion fruit juice were put into glass flasks (250 mL) and inoculated with the examined cultures (initial viable count approximately 9.00 ± 0.15 Log CFU/mL). Simulation in these flasks was performed continuously in phases mimicking mastication and conditions in the esophagus-stomach, duodenum, and ileum. Mechanical agitation was used to simulate the peristaltic movements, and the test was performed in an incubator shaker (Thermo Scientific, MaxQ 4000, USA) at 37 °C with rotation adjustment in each phase. Mastication was simulated using a saliva solution prepared with 100/mL α-amylase and diluted in 1 mM $CaCl_2$, whereas 1 M $NaHCO_3$ was used to adjust pH to 6.9; the simulated saliva was added at a rate of 0.6 mL/min for 2 min at 200 rpm. The esophagus-stomach conditions with 25 mg/mL pepsin diluted in 0.1 M HCl, added at a rate of 0.05 mL/mL, pH with gradual decrease using 1 M HCl (pH 5.5/10 min, pH 4.6/10 min, pH 3.8/10 min, pH 2.8/20 min, pH 2.3/20 min, and pH 2/20 min) under stirring (130 rpm). Duodenal conditions were simulated with 2 g/L pancreatin and 12 g/L bovine bile salts, diluted in 0.1 M $NaHCO_3$, pH adjusted for 5 with 0.1 M $NaHCO_3$, and exposure time of 30 min under stirring (45 rpm). Finally, ileal conditions were simulated with pH adjusted to 6.5 using 0.1 M $NaHCO_3$, exposure time of 60 min under stirring (45 rpm). All enzymes' solutions were filtered with sterilized syringe filters (0.22 µm pore size; Kasvi, Brazil) before use. All enzymes and bovine bile salts were purchased from Sigma-Aldrich (St. Louis, USA). After each simulated gastrointestinal condition, viable cells were determined.

2.4. Microbiological analysis

Microbiological analysis, growth media, and incubation conditions were performed according to Szutowska et al. (2020), with modifications. Serial dilutions of fermented juices were performed using sterile peptone water (0.1% w/v), and aliquots of appropriate dilutions were plated in duplicated by spread plate method. LAB were enumerated on MRS (Kasvi, Italy) agar after plates were incubated at 37 °C for 48 h under aerobic condition. The LAB survival rate was calculated as Eq. (1):

$$\text{Survival}(\%) = \text{final}(\text{CFU}/\text{mLNt}) / \text{initial}(\text{CFU}/\text{mLN0}) \times 100 \quad (1)$$

where Nt represents the CFU/mL at time t = 7, 14, 21 or 28 days of storage and N0 represents the CFU/mL at time t = 0 day.

2.5. Fluorescence microscopy

Cell images were obtained by fluorescence microscopy, according to Batista et al. (2019). The fluorescence analyses were made with the Zeiss Axio-Observer Z1 LSM 780 laser confocal microscope and Zen 2010 software (Carl Zeiss Microscopy MBH) at the Electron Microscopy Laboratory at the Federal University of Lavras. Syto9 fluorochrome (Thermo Fischer®) was used to label live bacterial cells (ChS1 detector), and a propidium iodide (PI) solution (Sigma®) was used to mark the dead bacteria (Ch2 detector). The nonfluorescent images were observed with the TPMT ChD detector. In brief, 5 µl of each sample was deposited above 0.25 cm^2 water-agar blocks and kept still for 5 min. On the same surface, 5 µl Syto9 (20 µM) was applied, and the mixture was incubated in the dark for 40 min. Next, 5 µl PI (1 µg/ml) was incubated for 5 min in the dark. The images were edited with FIJI ImageJ and Corel Draw software.

2.6. Color and pH determination

The juices' objective surface color was evaluated using a Konica Minolta CR10 (Tecnal, BR) colorimeter. The color was determined by direct reading of the reflectance of the color parameters L*, a*, b*. Numerical values of L*, a*, and b* were converted into ΔE* (total color difference), C* (chroma), and hue angle (h°) according to Eq. (2)-(4), respectively. Color measurements were taken in triplicate.

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad (2)$$

$$C^* = \sqrt{(a^*)^2 + (b^*)^2} \quad (3)$$

$$h^\circ = \tan^{-1}(b^*/a^*) \quad (4)$$

The juices pH was determined by direct measurement in a Digimed DM-22 potentiometer.

2.7. Volatile organic compounds analysis by GC-MS

Qualitative analysis of volatile organic compounds (VOC) in juices was performed on a headspace solid-phase microextraction (HS-SPME) gas chromatography-mass spectrometry GCMS Shimadzu QP-2010 SE, using Carbowax 20M column (30 m × 0.25 mm ID × 0.25 µm film). An aliquot of 3.0 mL of juices samples was added to a 20 mL vial and equilibrated at 60 °C for 15 min.

An automatic SPME holder (Supelco, Bellafonte, PA, USA) with a DVB/CAR/PDMS (50/30 μm) of 2 cm length fiber was used to capture the volatile compounds. The SPME fiber was exposed to the sample headspace at constant depth for 30 min. The temperature was kept at 60 °C throughout the extraction of the volatile compounds. After the extraction, the volatiles were directly desorbed on the GC liner and maintained at 230 °C for 2 min for fiber reconditioning. The carrier gas was helium at 1.0 $mL.min^{-1}$. The temperature ramp was: 60 °C for 5 min, increased to 230 °C at 10 $°C.min^{-1}$. The temperature was held at 230 °C for 15 min. The eluted volatile compounds were identified by matching their mass spectra to the NIST11 library. An alkane series (C10–C40) was used to calculate the retention index (RI) for each compound and compared them to the scholarly literature's RI values.

2.8. Sensory analysis

A Flash Profile (FP) was carried out according to the protocol described in Dairou and Sieffermann (2002). The 22 assessors, 6 male, and 16 female, aged from 23 to 53, were recruited from staff, undergraduate and postgraduate students at the Federal University of Lavras. All had broad experience in sensory evaluation and had participated in previous studies of different food matrices. The assessors met in three sessions. Three coded samples (two fermented and one unfermented juice) were presented simultaneously during the first session. The assessors were asked to individually generate the vocabulary to describe the essential attributes, which should be sufficiently discriminant to differentiate the samples concerning the appearance, flavor (taste), texture, aroma, and aftertaste. In the second session, they proceeded to the evaluation to rank all samples on their attributes according to intensity differences on an ordinal scale anchored from ‘lower’ to ‘higher’ (ties were allowed). Session 3 was a replicate of the evaluation step. The juices (40 mL) were served at consumption temperature (4.0 ± 1.0 °C), and mineral water was provided for rinsing the mouth between samples. The products served in the sensorial analysis were previously submitted to microbiological analysis according to the current legislation. This project was approved by the UFLA Ethics Committee (CAAE: 22480719.5.0000.5148).

2.9. Statistical analysis

All the treatments and assays were carried out thrice, and results were presented as mean ± standard error. The analysis of variance (ANOVA) was performed, and the Tukey test was used to calculate significant differences at $p < 0.05$. The analyzes were performed using Statistica software version 10.0 (Statsoft, USA).

A consensus configuration for the FP data was provided by generalized Procrustes analysis (GPA). Only attributes cited by at least four assessors (20% of the panel) were used to visualize the relationships between attributes and juices. All-Flash Profile data were performed by the XLSTAT® 2020.4.1 software (Addinsoft™, Paris, France).

3. Results

3.1. Viability of LAB in passion fruit juice during exposure to simulated gastrointestinal conditions

The viability of the two bacterial strains tested in the MRS (control) and FJ matrices in the simulated gastrointestinal model is showed in Table 1. There was no decrease in the bacterial viability throughout the simulated mouth conditions in both evaluated matrices. When exposed to the simulated esophagus and stomach conditions, the single culture CCMA 0743 kept its viable cell numbers in PF until 72 min of exposure and then decreased about 2 log cycles upon exposure to pH 2. A decrease in its viable cell numbers occurred (< 1 log cycle, for both MRS and FJ matrices) when exposed to duodenum conditions. This new level of cell viability was essentially maintained during the simulated ileum.

Table 1. Conditions used during the simulated digestion and the resultant viable cell counts of LAB strains in fermented passion fruit juice (FJ) and MRS broth.

Organ	Condition	Stirring (rpm)	pH	Time (min)	Viable cell counts (log CFU/mL)			
					CCMA 0743 (FJ)	CCMA 0743 + LBC-81 (FJ)	CCMA 0743 (MRS)	CCMA 0743 + LBC-81 (MRS)
Before simulation	-	-	-	-	9.04 ± 0.06^{aA}	9.02 ± 0.06^{aA}	8.87 ± 0.04^{aA}	8.87 ± 0.10^{aA}
Mouth	Saliva	200	6.9	2	8.87 ± 0.05^{aA}	8.64 ± 0.10^{aA}	8.73 ± 0.08^{aA}	8.64 ± 0.10^{aA}
Esophagus–Stomach	Pepsin	130	5.5	10	8.87 ± 0.09^{aA}	8.58 ± 0.07^{aA}	8.84 ± 0.08^{aA}	8.71 ± 0.10^{aA}
			4.6	10	8.90 ± 0.06^{aA}	8.91 ± 0.08^{aA}	9.02 ± 0.05^{aA}	8.75 ± 0.06^{aA}
			3.8	10	8.99 ± 0.07^{aA}	8.82 ± 0.04^{aA}	8.90 ± 0.06^{aA}	8.70 ± 0.08^{aA}
			2.8	20	8.65 ± 0.09^{aA}	8.61 ± 0.08^{aA}	8.58 ± 0.07^{aA}	8.50 ± 0.09^{abA}
			2.3	20	8.56 ± 0.05^{aAB}	8.66 ± 0.06^{aA}	8.51 ± 0.06^{aAB}	8.00 ± 0.21^{bB}
			2.0	20	6.87 ± 0.02^{bBC}	7.18 ± 0.02^{bB}	8.28 ± 0.07^{aA}	6.44 ± 0.48^{cC}
Duodenum	Pancreatin+ bile salt	45	5.0	30	6.08 ± 0.03^{cB}	5.83 ± 0.06^{cBC}	7.54 ± 0.10^{bA}	5.34 ± 0.08^{dC}
Ileum	-	45	6.5	60	6.18 ± 0.12^{cB}	5.77 ± 0.06^{cB}	7.62 ± 0.11^{bA}	5.07 ± 0.04^{dC}

Step 1: stage to simulate the conditions in the mouth; Steps 2 to 7: stages to simulate the conditions in the esophagus - stomach; step 8: stage to simulate the conditions in the duodenum; step 9: stage to simulate the conditions in the ileum. A-C: different superscript letters in the same row denote differences ($p < 0.05$) between the viable cell counts of LAB for the different treatments exposed to the same step of the simulated digestion, according to the Tukey's test. a–d: different capital letters in the same column denote differences ($p < 0.05$) between the viable cell counts for the same treatment exposed to the different step of the simulated digestion, according to the Tukey's test.

The assays inoculated with co-culture of LAB, as well as the single culture, during the exposure to esophagus and stomach conditions, occurred a decrease in viable cell numbers when pH reached 2.0 in the presence of pepsin, except for the single culture in MRS, which kept their population around 8.0 Log CFU/mL. On the other hand, the co-culture showed a smaller reduction when inoculated in PF. Finally, in the simulated duodenum conditions, viable cell counts of co-culture decreased around 3-4 log cycles in MRS and PF matrices compared to the initial situation.

Regarding the bacterial viability during the fermentation and storage period of FJ, high cell concentrations were observed in FJ. The strains maintained their survival above 8.0 Log CFU/mL during the refrigerated storage period (Fig. 1a). There was a significant decrease of bacterial viability on the 28th day of FJ storage for single CCMA 0743 culture, while by co-culture, the reduction occurred on the 14th day. However, the maximum reduction was less than 0.5 log CFU/mL at the end of shelf life. This reduction of 0.5 Log in microbial count represents, in absolute numbers, a decrease of approximately half of the total number of cells, as shown in Fig. 1b. Fig. 2 shows the viability of CCMA 0743 (Fig. 2ab) and co-culture (Fig. 2cd) in FJ at initial and final storage times measurements by fluorescence microscopy. As shown from the figure, the cells kept their viability (green bacilli) at the initial time and after the storage period (28 days). There was an increase in the number of dead cells (red bacilli), equivalent to approximately a 50% reduction from the initial total number of living cells.

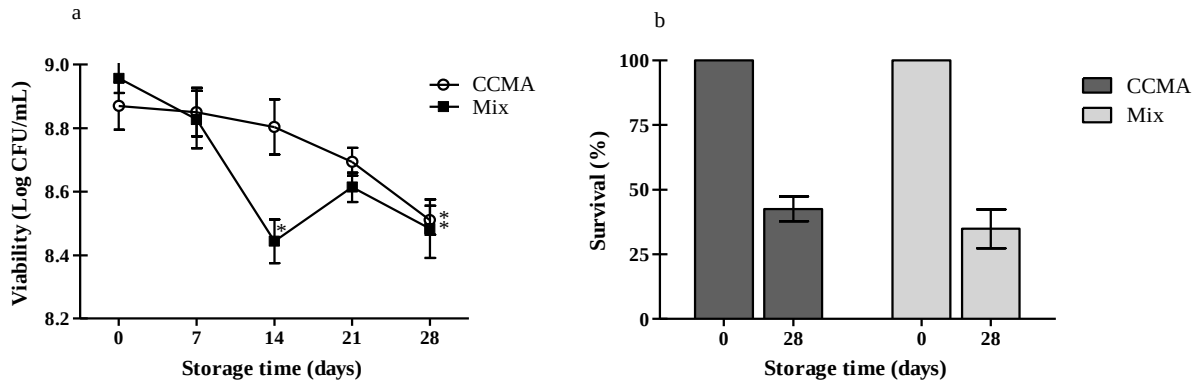

Fig. 1. Viability (a) and survival rate (b) of LAB in passion fruit juice fermented by *L. plantarum* CCMA 0743 (CCMA) and *L. plantarum* CCMA 0743 + *L. paracasei* LBC-81 (Mix) during storage at 4 °C. Bars indicate the error deviation. Asterisks, in the same fermented juice, mean values significantly different from the initial storage time (t = 0 days) according to the Tukey test at 95% confidence level.

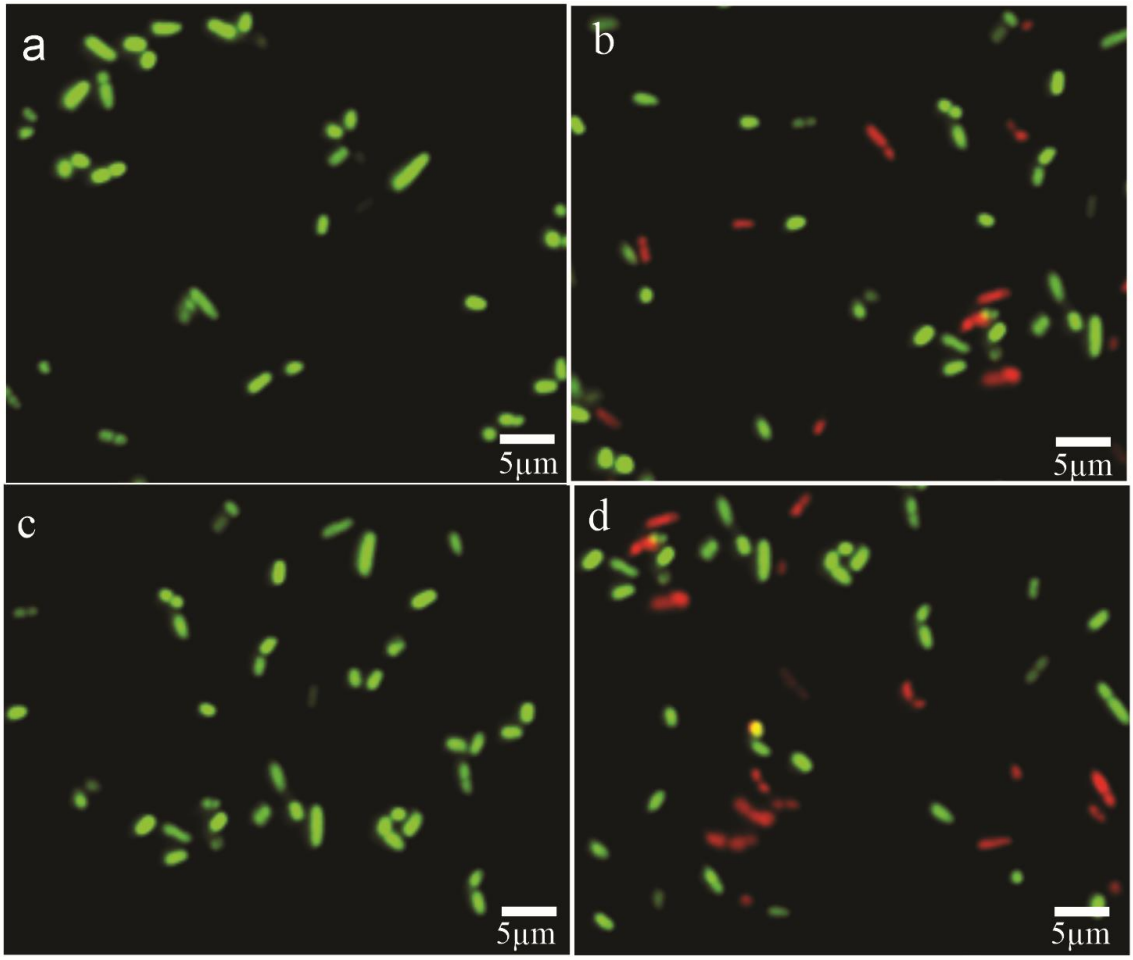

Fig. 2. Fluorescence microscopy of the passion fruit juices fermented by *L. plantarum* CCMA 0743 stored for 0 (a) and 28 (b) days and fermented by *L. plantarum* CCMA 0743 + *L. paracasei* LBC-81 stored for 0 (c) and 28 (d) days, at 4 °C.

3.2. Viability, color, and pH of fermented passion fruit juice during storage

The pH values decreased similarly among the assays (single and co-culture) throughout the 28 days of refrigerated storage (Table 2). At the end of the evaluated period, the pH values ranged from 3.51-3.65 for fermented juices. Color analysis results of fermented passion fruit juices were not presented difference ($p>0.05$) significantly, and chroma values ranged from 13.97-15.00. The hue angle (h°) showed a variation of approximately 2°, indicating that the juice's characteristic yellow was maintained throughout the storage period for both samples. Similarly, total color change ΔE^* of fermented passion juice was stable along the 28 days of refrigerated storage.

Table 2. Color parameters and pH of the fermented passion fruit juices after storage for 28 days at 4 ºC.

Treatment	Storage period (days)	Parameter			
		pH	C*	H°	ΔE*
CCMA 0743	0	4.02 ±0.02[a]	13.97 ±0.18[a]	88.37 ±0.64[a]	
	7	3.92 ±0.01[b]	14.07 ±0.07[a]	89.63 ±1.14[a]	0.64 ±0.13[a]
	14	3.92 ±0.03[b]	13.67 ±0.07[a]	89.13 ±1.26[a]	0.43 ±0.06[a]
	21	3.77 ±0.01[c]	14.27 ±0.03[a]	89.40 ±0.31[a]	1.63 ±0.26[a]
	28	3.65 ±0.02[d]	14.43 ±0.83[a]	90.40 ±0.91[a]	1.39 ±0.64[a]
CCMA 0743 +	0	3.96 ±0.02[a]	14.00 ±0.12[a]	88.77 ±0.09[a]	
LBC-81	7	3.86 ±0.01[b]	14.23 ±0.24[a]	88.47 ±0.52[a]	0.52 ±0.21[a]
	14	3.85 ±0.01[b]	14.53 ±0.03[a]	89.03 ±1.43[a]	1.19 ±0.14[a]
	21	3.64 ±0.02[c]	14.13 ±0.09[a]	89.67 ±0.18[a]	0.99 ±0.04[a]
	28	3.51 ±0.01[d]	15.00 ±0.23[a]	90.07 ±0.52[a]	1.34 ±0.21[a]

Means ± standard error in the same column followed by different lowercase letters indicate statistically significant differences at $p \leq 0.05$, according to Tukey test (n = 3), for each treatment affected by the storage time.
C* – Chroma, H° – Hue angle, ΔE* – total color difference.

3.3. Volatile organic compounds and sensory profiles of passion fruit juices

Unfermented passion fruit juice (UJ) and the juices fermented by single and co-culture (Mix) were characterized for the VOC profile. A total of 26 VOCs were identified in the three juice samples. The identified VOC were grouped according to chemical class: alcohols (9), aldehyde (1), ketones (4), acids (9), and esters (3) (Table 3). Among these groups, alcohols and acids accounted for 69.2% of total VOC. The results indicate that only one ketone, 2-butanone, 4-(2,2-dimethyl-6-methylenecyclohexyl), and one ester, citronellyl butyrate, were detected in all samples. Most ketones were detected only in the UJ sample, indicating that the fermentation could reduce the floral and fruity notes, as observed in the sensory analysis. On the other hand, regarding alcohols, the compounds 2,6-octadien-1-ol, 3,7-dimethyl-, (Z), and 1-dodecanol and only detected in fermented samples, which could have contributed to the increased characteristic fatty odor (not observed in sensory analysis), and floral and fruit flavors.

Table 3. Volatile compounds in passion fruit juices before and after fermentation by LAB cultures.

Chemical class	Volatile compounds	Sensory perception*	UJ	CCMA	Mix
Alcohol	1,6-Octadien-3-ol, 3,7-dimethyl	Spicy, citrus taste, floral, woody, sweet with a green, spicy tropical nuance	+	+	+

	α-Terpineol	Lime, peach, floral flavor, sweet, lime taste	+	+	+
	Citronellol	Floral	+	+	+
	2,6-Octadien-1-ol, 3,7-dimethyl-, (Z)	Floral, Fruit flavor	-	+	+
	Benzyl alcohol	Sharp burning taste	+	+	+
	5,9-Undecadien-2-ol, 6,10-dimethyl-		+	+	+
	1-Dodecanol	Characteristic fatty odor; fatty, waxy flavor	-	+	+
	Phenol, 2,4-bis(1,1-dimethylethyl)	Paint	+	+	+
	Ethanol, 2-(dodecyloxy)		+	+	+
Aldehyde	Benzaldehyde	Almond odor	+	+	+
Ketone	2-Butanone, 4-(2,2-dimethyl-6-methylenecyclohexyl)		+	+	+
	5,9-Undecadien-2-one, 6,10-dimethyl-, (Z)	Fruit flavor	+	-	-
	3,5,9-Undecatrien-2-one, 6,10-dimethyl-, (E,Z)	Floral flavor	+	-	-
	5,9-Undecadien-2-one, 6,10-dimethyl	Fruit flavor	+	-	-
Acid	Octanoic acid	Slightly sour taste	+	+	+
	n-Hexadecanoic acid	Faint oily aroma	+	+	+
	Octanoic acid, hexyl ester	Green, herb, and oil flavors	+	-	+
	Pentanoic acid, 4-methyl-, phenylmethyl ester		+	-	-
	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester		-	+	+
	Acetic acid	Strong odor of vinegar	+	+	+
	Hexanoic acid, 3-hexenyl ester, (Z)	Fruit, prune (odor)	+	+	+
	Hexanoic acid, hexyl ester	Apple peel, cut grass, and fresh taste	+	+	+
	Hexanoic acid	Fatty-rancid odor, acrid-acid	+	+	+
Ester	Methyl salicylate	Odor and taste of wintergreen	-	+	+
	Dibutyl phthalate	Strong and bitter tastes	+	-	-
	Citronellyl butyrate	Fruit	+	+	+

+, detected; -, not detected.

UJ, unfermented juice; CCMA, fermented juice by *L. plantarum* CCMA 0743; Mix, fermented juice by *L. plantarum* CCMA 0743 and *L. paracasei* LBC-81.

*Sensory descriptions were cited from https://pubchem.ncbi.nlm.nih.gov/, www.flavornet.org and https://www.femaflavor.org/.

The sensory analysis was performed for fermented and unfermented samples, and the results are showed in Table 4. Following FP's first session, the assessors used variation from 4 to 14 terms to describe the passion fruit juices and had generated 42 unique sensory attributes (Table 4). These were reduced to 16 terms used by at least 20% of the taste panel. In the task, four descriptors emerged as the most relevant to describe the samples. The most frequently mentioned attributes were “yellow color” (17), “sweet taste” (14), “acidic taste” (14), and “thick texture” (11), cited by at least 50% of the assessors.

Table 4. Attributes used by assessors in Flash Profile approaches.

	Attribute	**Frequency of mention**	**Attribute**	**Frequency of mention**	**Attribute**	**Frequency of mention**
Appearance	Yellow	17	Fruit pieces	1	Clarified	1
	Foaminess	9	Fresh juice	1	Orange	1
Aroma	Passion fruit	9	Citrusy	2	Fruit	1
	Sweet	7	Medicinal	1	Unripe fruit	1
	Acidic	4	Overripe	1	Herbaceous	1
	Fermented	3				
Taste	Sweet	14	Salty	4	Sweetener	3
	Acidic	14	Bitter	4	Caramel	2
	Passion Fruit	5	Astringent	4	Watery	1
	Umami	4	Fermented	3	Metallic	1
Texture	Thick	11	Sparkling	3	Creaminess	1
	Uniform	7	Grainy	1		
Aftertaste	Acidic	8	Umami	1	Passion fruit	1
	Sweetener	6	Sweet	1	Fermented	1
	Bitter	2	Fruity	1	Astringent	1

The GPA results from the passion fruit juice samples' consensual configuration and the attributes explained 100.0% of the total variability in two dimensions (87.31% for the first dimension and 12.69% for the second dimension). Among the 16 attributes that showed the highest frequency of citation, bitter taste and sweetener aftertaste displayed higher correlations (-0.983 and -0.832, respectively) with the second dimension. The other 14 attributes were correlated (> |0.800|) with the first dimension.

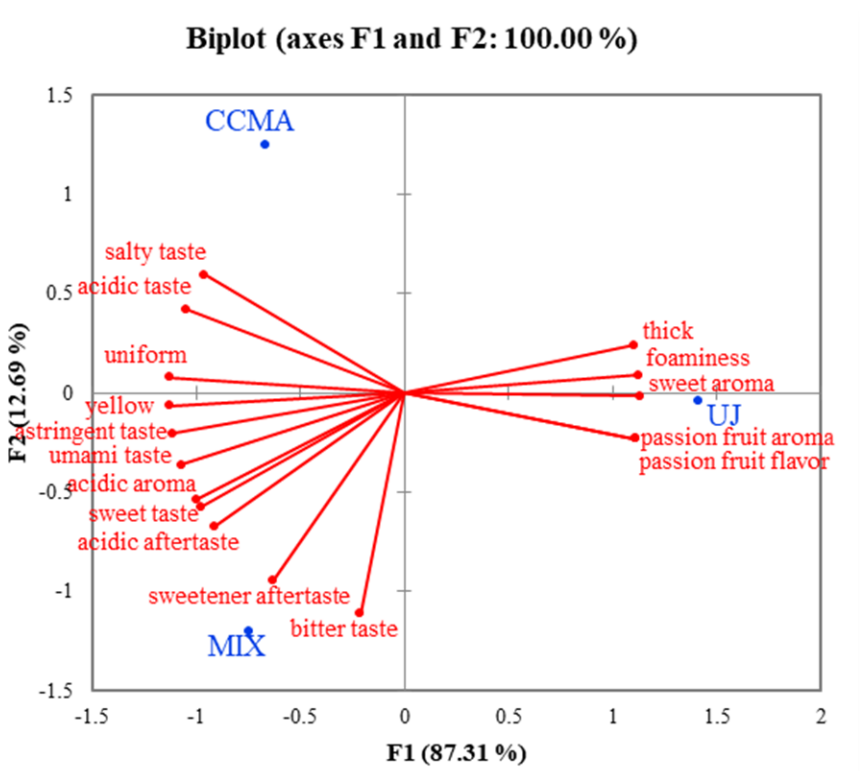

Fig. 3. Biplot of the three passion fruit juices, UJ (Unfermented Juice), CCMA (juice fermented by *L. plantarum* CCMA 0743) and MIX (juice fermented by *L. plantarum* CCMA 0743 + *L. paracasei* LBC-81) and the terms used to describe the samples at the first two dimensions of the general Procrustes analysis (GPA) of data from flash profile. Only attributes cited by at least 20% on the panelist were used.

The UJ sample is projected on the right part of the FP plot in Fig. 3, indicating that it was described mainly with the terms “thick”, “foaminess”, “sweet aroma”, “passion fruit aroma,” and “passion fruit flavor”, which are exclusive attributes for this juice. The fermented passion fruit juices were located on the left, indicating these samples were significantly different from UJ. For the CCMA sample (negative in F1 and positive in F2), the correlated terminology was “salty taste” and “acidic taste”. Meanwhile, the co-culture (MIX) sample was mainly described as “bitter taste” and “sweetener aftertaste”. GPA analysis showed that fermented juices were mainly discriminated by the lower intensity of passion fruit notes than the unfermented juice. This notes might be linked with higher amount of ketone compounds in unfermented juice.

4. Discussion

The health benefit of probiotics relies upon their concentration in foods during storage and their ability to survive the gastrointestinal tract's adverse conditions (Perricone et al., 2015). The stress conditions imposed by the gastrointestinal environment, whether due to low pH, presence of digestive enzymes and bile salts, can negatively affect the survival of microorganisms. In contrast, the food matrix can contain substances that can confer protective effects on bacterial cells (Lopes et al., 2020; Madureira et al., 2011). At the beginning of digestion, all cultures were at levels of approximately 10^9 CFU/mL. Although the definition of probiotics does not include a reference to a specific dose, it states that probiotics should be administered in amounts that are adequate to result in a health benefit for the host (Binda et al., 2020). Thus, if a particular study elicits the desired health benefit, then that dose would serve as the minimum dose for which a health claim should be permitted. It means that the CFU per serving of a probiotic should be that which can be guaranteed through the end of shelf life and matches the dose used in human studies demonstrating a health benefit (Binda et al., 2020; Sanders et al., 2018). The exposure to the seventh and eighth digestive phases (stomach conditions, pH 2.0, and duodenum conditions, respectively) caused sharp decreases in viable counts of all examined cultures, except CCMA 0743 in MRS broth, that presented viable count reduction only in the duodenum conditions (pancreatin + bile salts, pH 5.0). De Albuquerque et al. (2018) reported higher protective effects on bacterial cells of *L. paracasei* and *L. plantarum* strains when exposed to simulated gastrointestinal conditions in MRS broth than in grape juice. The nature of a food matrix could affect the probiotic viability during GIT, and the presence of organic acids and phenolic compounds in passion fruit juice can cause a high impact on the viable cell counts (Ranadheera et al., 2010). Herein, we use commercial and potential probiotic strains that survived at suitable counts (~ 6.0 Log CFU/mL) at the end of the process. In this sense, it is essential to evaluate the food matrix's bacterial behavior under gastrointestinal conditions since ecological interactions are strain/matrix dependent.

In general, fermented foods with live or active cultures, including probiotics, must contain minimum probiotic cell viability per serving, as previously mentioned. Considering that a portion of juice usually has at least 100 mL, thus, both juices elaborated in the present work contained high LAB viable cell counts throughout the 28 days of the storage period. Moreover, the present study results corroborate some reports (Fonteles et al., 2013; Valero-Cases & Frutos, 2017; Zhao et al., 2019) of the efficiency of the plant matrix to maintain the high bacterial viability during storage. In contrast, (Espirito-Santo et al., 2015) reported a variable decrease of lactobacilli strains in apple juices on day 28, suggesting that adaptation to the fruit juices' stressful conditions are specific-strains. Cell viability can be obtained by either the plating method or fluorescence microscopy. The fluorescence microscopy analysis discriminates between live and dead cells, which can be observed, respectively,

by the emission of fluorescence at different wavelengths (Batista et al., 2019). Maukonen et al. (2006) consider that fluorescence microscopy and plate count culture methods agree with each other, rendering fluorescent techniques suitable alternatives for the rapid viability assessment of probiotic products. In the present study, the methodologies corroborate each other and present themselves as reliable tools in verifying the number of bacterial cells, exceptionally viable ones.

According to Perricone et al. (2015), juice pH is one of the most critical factors affecting probiotics' survival. The low pH increases the concentration of undissociated organic acids in the juices, showing antimicrobial effects. The observed decrease of pH (0.45-unit maximum) in the juices during refrigerated storage could be regarded as the result of the sum of the different post acidification capacities of the strains and the substrate's buffer power. This pH reduction significantly impacted cell viability, corroborating with work performed by Fonteles et al. (2013). These authors demonstrated that changes in the product pH during refrigerated storage could influence microbial survival, associated with the adaptation phase to the adverse condition imposed by low pH.

Pasteurization promotes enzyme inactivation, which contributes to the characteristic color maintenance. This heat treatment (80 °C/5 min) may have contributed to its stability, thus ensuring more excellent microbiological safety and standardization during the storage period. First, the yellow color intensity is maintained is consistent with the values of a*, and b* parameters kept at neutral and low positive values, respectively (data not shown). Second, the chroma parameter is associated with the color intensity. Thus, the yellow intensity of passion fruit juice did not modify after fermentation. The hue angle obtained for the fermented juices was about 89°, representing the characteristic color of passion fruit pulp (Dias et al., 2017). Finally, observed stable yellow color could be seen by the total color change (ΔE values), that was not exceeded the threshold for the human eye perception ($\Delta E<2.0$) (Pereira et al., 2011). This result is positive since beverage color is an important quality parameter, and its stability in juices can affects the juice’s acceptability by consumers (Pimentel, Madrona, Garcia, et al., 2015).

The volatile aromatics are considered essential food products for consumer sensory acceptability (Kaprasob et al., 2017). Notably, positive sensory perceptions as floral and fruit notes were primarily found in all samples, mainly unfermented juice. Regarding the acid compounds detected in both three juice samples, acetic and octanoic acids have demonstrated antimicrobial function (Kinderlerer & Lund, 1992; Krusong et al., 2020) in addition to providing a sour taste. Hexanoic acid, 3-hexenyl ester (Z), and hexanoic acid hexyl ester have been reported as the main volatile compound that gives passion fruit its distinctive aroma (Macoris et al., 2011).

Aroma-forming ability is strain-dependent and is related to the metabolic variability of different strains used in lactic acid fermentation. Single or mixed species starters exert different roles

in modifying the substrates and may contribute better and certain quality characteristics related to aroma-flavor features (Cui et al., 2019; Ricci et al., 2018). For example, there was detection of the octanoic acid, hexyl ester compound only in the co-culture fermentation juice (Table 3), corroborating with the previous statements in the present study.

UJ contains general fruity characteristics at quite different intensity levels from the fermented samples from the sensory point of view. The fermentation process, characterized by bacterial growth in the beverage, may have contributed to the aroma and flavor decrease of the passion fruit due to compounds' consumption and production (Ellendersen et al., 2012). About other attributes, sweetness is generally thought of as a well-shared and consensual basic taste characteristic (Veinand et al., 2011) and presents naturally high negative correlations with acidity (Carbonell et al., 2007). However, in the present study, the assessors characterized the unfermented passion fruit beverage as less sweet and less acidic, although the added sweetener's concentration was the same for both samples. According to Veinand et al. (2011), taste-flavor interactions may also contribute to differences in the sensory description and, consequently, may have generated a positive correlation between sweetness and acidity. Therefore, our results corroborate Pimentel, Madrona, and Prudencio (2015)'s findings, which the pure juice was characterized by fruit flavor and aroma and lower sweetness.

Meanwhile, the passion fruit juice fermented by CCMA 0743 strain was described as the sample with a salty taste. Some undesirable descriptors were mentioned, such as bitter taste, probably due to stevia sweetener, reported by Rocha and Bolini (2015). In another study, Luckow and Delahunty (2004) reported that descriptive analysis revealed nontypical aroma and tastes in blackcurrant juice with added probiotic *L. plantarum*.

The advantage of the simultaneous presentation of products does not need any familiarization phase with the subjects' samples. From the start, they can generate attributes that discriminate and are relevant for the product (Bredie et al., 2017). The sensory evaluation generated attributes are similar to the trained panel for the passion fruit juice sample (Rocha & Bolini, 2015). In this study, Flash Profile was efficient for describing the passion fruit juice characteristics sensory, in the compilation of terminology, with terms strongly linked to the product's processing. Lastly, the formation and degradation of aroma compounds probably contributed to the fermented samples' sensory profile difference. Therefore, the evaluation of volatile compounds is an important strategy for linking sensory characteristics and defining product quality.

5. Conclusion

Our results indicate that the potentially probiotic *L. plantarum* CCMA 0743 strain, single or co-culture, confers unique sensory characteristics to passion fruit juice. Moreover, this strain presented the ability to survive to simulated gastrointestinal conditions and refrigerated storage period in the product. Despite this, the product color kept stable during the shelf life, contributing to consumer acceptance.

The fermentation of passion fruit juice through evaluated LAB strains may be an exciting option to offer probiotic bacteria. However, other technologies, such as non-thermal conservation methods and the addition of prebiotics, can be used concurrently with fermentation to avoid nutritional losses and achieve better organoleptic and viability results. Based on the obtained results, it can be concluded that juice fermentation is affected by the interactions of individual strains or strain-strain interactions with the food matrix, demonstrating that physical-chemical, sensory and functional assessments are necessary for developing new products with acceptable characteristics.

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