UNIVERSIDADE CEUMA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIODIVERSIDADE
E BIOTECNOLOGIA DA REDE BIONORTE

EFEITOS DOS SUCOS DE FRUTAS DO CERRADO MARANHENSE NA VIABILIDADE DE CEPAS PROBIÓTICAS E EM MODELOS DE ENDOTOXEMIA EXPERIMENTAL

ADRIELLE ZAGMIGNAN

São Luís – MA

2020

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Tese de doutorado apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Biodiversidade e Biotecnologia da Rede BIONORTE, na Universidade CEUMA, como requisito parcial para a obtenção do Título de Doutor em Biotecnologia.

Orientador(a): Prof. Dr. Luís Cláudio Nascimento da Silva

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DEZEMBRO/2020

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Zagmignan, Adrielle.
EFEITOS DOS SUCOS DE FRUTAS DO CERRADO MARANHENSE NA VIABILIDADE DE CEPAS PROBIÓTICAS E EM MODELOS DE ENDOTOXEMIA EXPERIMENTAL / Adrielle Zagmignan. - 2020.
91 f.

Orientador(a): Luís Cláudio Nascimento da Silva.
Tese (Doutorado) - Programa de Pós-graduação em Rede - Rede de Biodiversidade e Biotecnologia da Amazônia Legal/ccbs, Universidade Federal do Maranhão, SAO LUIS, 2020.

1. Endotexemia. 2. Lactobacillus. 3. Suco de frutas. I. Nascimento da Silva, Luís Cláudio. II. Título.

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Aprovada em _____/_____ /_____

Banca examinadora

_____

Profº. Drº. Luís Cláudio Nascimento da Silva
Orientador – Presidente da banca

_____

Profª. Drª. Wolia Costa Gomes
Universidade CEUMA – Examinador externo

_____

Profª. Drª. Cristina de Andrade Monteiro
Instituto Federal de Educação, Ciência e Tecnologia do Maranhão – IFMA
Examinador externo

_____

Profª. Drª. Julliana Ribeiro Alves dos Santos
Universidade CEUMA – Examinador interno

_____

Profº Drº Eduardo Martins de Sousa
Universidade CEUMA – Examinador externo

RESUMO

ZAGMIGNAN, Adrielle. **Efeitos dos sucos de frutas do cerrado maranhense na viabilidade de cepas probióticas e em modelos de endotoxemia experimental**. 2020. 69 f. Tese (Doutorado em Biotecnologia-Rede Bionorte) – Universidade Ceuma, 2020.

A endotoxemia é uma condição clínica com riscos ao indivíduo, caracterizada por uma resposta sistêmica severa que causa disfunção de múltiplos órgãos e representa um sério problema para os cuidados com o sistema de saúde em todo o mundo. Algumas evidências mostraram fortes efeitos imunomoduladores induzidos por bactérias probióticas em modelos experimentais de distúrbios inflamatórios. Podendo ser associado ao uso de frutas como veículo para o crescimento dessas bactérias probióticas. Nesse contexto, destaca-se a ingestão de suco fermentado com *Lacticaseibacillus rhamnosus* (*L. rhamnosus*) por camundongos submetidos à endotoxemia induzida por lipopolissacarídeos (LPS). Após otimizar a melhor condição de cultivo para o *L. rhamnosus* em diferentes concentrações de polpa no suco de cupuaçu, além de melhor produção de ácido lático, camundongos C57BL / 6 (n = 12 / grupo) foram alocados aleatoriamente em grupos experimentais que receberam doses orais (100 µL / camundongo) de solução salina tamponada com fosfato (PBS), suco de cupuaçu não fermentado ou suco de cupuaçu fermentado por *L. rhamnosus* (resultando em 10^8 UFC / mL) por cinco dias. Cada animal foi colocado em uma gaiola individual e recebeu uma injeção intraperitoneal de LPS e foi sacrificado após 6 h e 120 h após a inoculação com LPS. A severidade da endotoxemia também foi avaliada diariamente. Todos os dias, o peso corporal e a temperatura foram registrados e comparados com os dados obtidos antes da inoculação do LPS. Os órgãos de cada animal foram pesados e medidos, além da realização da punção do sangue cardíaco e coleta do lavado peritoneal para contagem de células totais e diferenciais. Os principais resultados obtidos sugerem que a administração de suco fermentado por *L. rhamnosus* reduziu a gravidade da endotoxemia mediada por LPS, reduziu a perda de peso, alterou a migração de células para a cavidade peritoneal e reduziu o aumento de células no sangue. Em conclusão, nossos dados mostram que a terapia precoce do suco de cupuaçu fermentado com *Lacticaseibacillus rhamnosus* ATCC 9595 pode reduzir a inflamação sistêmica em um modelo experimental de sepse em camundongos.

Palavras-chave: *Lactobacillus.* Endotexemia. Suco de frutas.

ABSTRACT

ZAGMIGNAN, Adrielle. **Efeitos dos sucos de frutas do cerrado maranhense na viabilidade de cepas probióticas e em modelos de endotoxemia experimental**. 2020. 69 f. Tese (Doutorado em Biotecnologia-Rede Bionorte) – Universidade Ceuma, 2020.

Endotoxemia is a life-threatening clinical condition, characterized by an impaired systemic response that causes multiple organ dysfunction and represents a serious problem for care with the health system worldwide. Some evidence has shown strong immunomodulatory effects induced by probiotic bacteria in experimental models of inflammatory disorders. It is associated with the use of fruits as a vehicle for the growth of these probiotic bacteria. In this context, the intake of fermented juice of *Lacticaseibacillus rhamnosus* (*L. rhamnosus*) in mice submitted to lipopolysaccharide-induced endotoxemia (LPS). After optimizing the best cultivation condition for *L. rhamnosus* in different pulp concentrations in cupuaçu juice, in addition to better production of lactic acid, the C57BL / 6 mice (n = 12 / group) were randomly allocated to experimental groups that received doses oral (100 µL / mouse) of phosphate buffered saline (PBS), unfermented cupuaçu juice or cupuaçu juice fermented by *L. rhamnosus* (resulting 10^8 CFU / mL) for five days. Each animal was placed in an individual cage and received an intraperitoneal injection of LPS and was sacrificed after 6 h and 120 h after inoculation with LPS. The severity of endotoxemia was also daily evaluated. Every day, body weight and temperature were recorded and compared with data obtained before inoculation of LPS (baseline). The organs of each animal were weighed and measured, in addition to the puncture of cardiac blood to measure total and differential cells. The main results obtained suggest that the administration of *L. rhamnosus*-fermented juice reduced the severity of LPS-mediated endotoxemia, reduced weight loss, amended the migration of cells to peritoneal cavity and reduced the increase of cells in blood. In conclusion, our data show that early therapy of fermented cupuaçu juice with *Lacticaseibacillus rhamnosus* ATCC 9595 can reduce systemic inflammation in an experimental model of sepsis in mice.

Key-words: *Lactobacillus*. Endotexemia. Fruit juice.

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SUMÁRIO

1. Introdução

O lipopolissacarídeo (LPS) é um componente estrutural da membrana externa das bactérias Gram-negativas (KAMISOGLU; HAIMOVICH; CALVANO; COYLE *et al.*, 2015) que podem ser reconhecidos pelo receptor 4 (TLR-4) (BERTANI; RUIZ, 2018; COCHET, FLORENT; PERI, FRANCESCO, 2017). Níveis elevados de LPS na corrente sanguínea são classificados como endotoxemia, que pode resultar de bacteremia e / ou disfunções metabólicas (SATOKARI, 2020). A endotoxemia metabólica está intimamente relacionada a alterações na homeostase da microbiota intestinal, como consequência da obesidade, lesão hepática ou outras condições crônicas relacionadas à inflamação (FUKE, N.; NAGATA, N.; SUGANUMA, H.; OTA, T., 2019; MOLUDI; MALEKI; JAFARI-VAYGHYAN; VAGHEF-MEHRABANY *et al.*, 2020; SATOKARI, 2020).

Independentemente de suas causas, a endotoxemia é uma condição clínica que conferem riscos ao indivíduo, caracterizada por uma resposta sistêmica prejudicada que provoca a disfunção de múltiplos órgãos e representa um problema sério para os sistemas de saúde em todo o mundo (KOUTROULIS; BATABYAL; MCNAMARA; LEDDA *et al.*, 2019; VAN DER POLL; VAN DE VEERDONK; SCICLUNA; NETEA, 2017). Devido à complexidade da fisiopatologia do choque por endotoxinas, o desenvolvimento de uma terapia eficaz ainda é um grande desafio (CAVAILLON; SINGER; SKIRECKI, 2020; VAN DER POLL; VAN DE VEERDONK; SCICLUNA; NETEA, 2017).

Algumas evidências mostraram fortes efeitos imunomoduladores induzidos por bactérias probióticas (particularmente certas cepas de *Lactobacillus* sp.) em modelos experimentais de distúrbios inflamatórios (ABDO; LECUREUX; LAVOY; EKLUND *et al.*, 2019; SIRESWAR; BISWAS; DEY, 2020; VAREILLE-DELARBRE; MIQUEL; GARCIN; BERTRAN *et al.*, 2019). Esses resultados advogam seu possível uso alternativo no tratamento das condições clínicas relacionadas à endotoxemia (AVILA; MICHELS; VUOLO; BILESIMO *et al.*, 2020; HARO; MONACO; MEDINA, 2018). Essas bactérias podem modular a resposta do hospedeiro devido à estimulação direta das células imunes e / ou modificação da microbiota intestinal (AVILA; MICHELS; VUOLO; BILESIMO *et al.*, 2020; MAO; QI; CUI; DOU *et al.*, 2020; MAZZEO; LUONGO; SASHIHARA; ROSSI *et al.*, 2020; NATION; DUNNE; JOSEPH; MENSAH *et al.*, 2017; VILLANOVA; MENEGHELLI; DANTAS, 1987).

Lactobacillus são bactérias ácido láctico que são habitantes normais do intestino humano e também são consumidas como alimento por um longo período de tempo (SOUSA;

RAMA; VOLKEN DE SOUZA; GRANADA, 2020). No entanto, a maioria dos produtos que contêm *Lactobacillus* são derivados do leite, impondo um obstáculo para alguns indivíduos (como aqueles com intolerância à lactose, alergia às proteínas do leite e veganos), apontando para a importância do material derivado de plantas para a obtenção de novos produtos probióticos (KIM; CHOI; PARK; KIM, 2019). Nesse sentido, o uso de suco como veículo para essas bactérias probióticas está surgindo uma alternativa adequada (KIM, 2017; KWAW; MA; TCHABO; APALIYA *et al.*, 2018). Além disso, a disponibilidade de vários compostos bioativos em sucos pode atuar em sinergia com o *Lactobacillus* para promover os efeitos benéficos para os consumidores (BANCALARI; CASTELLONE; BOTTARI; GATTI, 2020; LIU; CHEN; CHEN; ZHONG *et al.*, 2018).

O suco de *Theobroma grandiflorum* (Cupuaçu ou Cupuaçu) e *Platonia insignis* Mart. (Clusiaceae) (Bacuri) são muito apreciados no Brasil *in natura* ou na preparação industrial para a produção de sucos, sorvetes, picolés, geleias, chocolates, doces, sendo uma importante fonte de vitaminas e minerais (PUGLIESE; TOMAS-BARBERAN; TRUCHADO; GENOVESE, 2013) e possui propriedades antioxidantes, como vitaminas C (ácido ascórbico) e E (tocoferóis), flavonóides, antocianinas e polifenóis, além de sacáridos (glicose, frutose e sacarose) e minerais (como Na, K, Ca, Mg, P, Fe, Zn e Cu) (DE HOLANDA; CLÍMACO; HUNALDO; GOMES *et al.*, 2020; DE MORAES BARROS; GARCÍA-VILLALBA; TOMÁS-BARBERÁN; GENOVESE, 2016; PUGLIESE; TOMAS-BARBERAN; TRUCHADO; GENOVESE, 2013).

2. Revisão bibliográfica

2.1. Lipopolissacarídeo (LPS) e a indução de endotoxemia

O lipopolissacarídeo (LPS) é um glicolipídio cuja principal função é servir como componente estrutural da parede celular de bactérias Gram-negativas, proporcionando uma potente barreira contra algumas substâncias que podem atravessar a bicamada lipídica bacteriana, favorecendo que estes microrganismos tornem-se naturalmente resistentes a muitos compostos antimicrobianos (UPPU; KONAI; SARKAR; SAMADDAR *et al.*, 2017; ZHANG; MEREDITH; KAHNE, 2013). Embora ocorra variações na formação do LPS nas espécies bacterianas, sua estrutura geral é conservada (WHITFIELD; TRENT, 2014). Desse modo, sua estrutura é formada por três componentes, que são, o lipídio A, constituído por cadeias hidrofóbicas de hidrocarbonetos, localizado externamente a membrana plasmática; enquanto a região oligossacarídica e o antígeno O, consistem em estruturas hidrofílicas localizadas na superfície da membrana celular (BOTOS; NOINAJ; BUCHANAN, 2017).

Evidentemente, o LPS desempenha um papel crucial nas interações bactéria e hospedeiro modulando as respostas do sistema imunológico do indivíduo (BERTANI; RUIZ, 2018), ação promovida principalmente pela presença do lipídio A (GOMES; COSTA; ALFENAS, 2017). O reconhecimento desta endotoxina pode desencadear uma resposta descontrolada conhecida como Síndrome da Resposta Inflamatória Sistêmica (SIRS, do inglês *Systemic Inflammatory Response Syndrome*) (BOUTAGY; MCMILLAN; FRISARD; HULVER, 2016; CANI; AMAR; IGLESIAS; POGGI *et al.*, 2007; GNAUCK; LENTLE; KRUGER, 2016). Esta condição clínica incluí choque, coagulação intravascular disseminada, além da falência de múltiplos órgãos, resultando em altas taxas de morbidade e mortalidade (VAN LIER; GEVEN; LEIJTE; PICKKERS, 2019).

O LPS pode atingir a circulação sanguínea como parte integrante da bactéria, caracterizando o estado de sepse, de forma direta ou através da disseminação a partir de uma infecção local no tecido cutâneo, trato respiratório ou gastrointestinal (LELUBRE; VINCENT, 2018). Nestes casos, a endotoxina alcança o sangue através da via linfática, especialmente através dos epitélios mucoso do trato respiratório superior e trato gastrointestinal, especificamente boca e cólon (BOUTAGY; MCMILLAN; FRISARD; HULVER, 2016). Em adição, o LPS presente nas secreções orais e nos alimentos e bebidas contaminados podem mover-se para o sangue por meio da via linfática do intestino delgado, geralmente associados com lipídios ricos em triglicerídeos (MUNFORD, 2016).

Tem-se evidenciado que dietas ricas em gorduras favorece o predomínio de bactérias Gram-negativas em relação a bactérias Gram-positivas na composição da microbiota intestinal, favorecendo aumento da permeabilidade intestinal por meio da liberação de mediadores inflamatório, tais como fator de necrose tumoral TNF-α, interleucina-1 (IL-1) e espécies reativas de oxigênio e nitrogênio (CANI; BIBILONI; KNAUF; WAGET *et al.*, 2008; FUKE, NOBUO; NAGATA, NAOTO; SUGANUMA, HIROYUKI; OTA, TSUGUHITO, 2019; HASAIN; MOKHTAR; KAMARUDDIN; ISMAIL *et al.*, 2020; WISNIEWSKI; DOWDEN; CAMPBELL, 2019). Esses mediadores facilitam a migração de LPS para a corrente sanguínea o que leva a indução da endotoxemia sistêmica (KODURU; ACHUTHANKUTTY; GHANIM; DANDONA, 2012). Dietas rica em gorduras podem desencadear endotoxemia metabólica que possivelmente levam ao desenvolvimento de obesidade e resistência à insulina (ANDRÉ; LAUGERETTE; FÉART, 2019; CANI; AMAR; IGLESIAS; POGGI *et al.*, 2007; HASAIN; MOKHTAR; KAMARUDDIN; ISMAIL *et al.*, 2020).

Compreende-se que uma infecção local ou limitada a um determinado sistema em resposta a endotoxina traz menos danos teciduais ao indivíduo, possibilitando a eliminação do agente agressor (MUNFORD, 2016). Por outro lado, a intensificação desse mecanismo de

defesa por elevados níveis de citocinas, atividade de espécies reativas de oxigênio e nitrogênio, podem trazer danos vasculares propiciando sepse (BERTANI; RUIZ, 2018; LOPEZ-COLLAZO; DEL FRESNO, 2013). É evidente que o sistema imunológico evoluiu para responder principalmente à característica mais conservada do LPS, a estrutura lipídica A. Contudo, a diversidade considerável nas estruturas de LPS, mesmo dentro do lipídio A como já mencionado, pode ser responsável por desencadear variáveis tipos de resposta imune do hospedeiro (BERTANI; RUIZ, 2018; BOTOS; NOINAJ; BUCHANAN, 2017).

Uma vez que o LPS alcança a circulação sanguínea, este antígeno é reconhecido por seu receptor de reconhecimento padrão, *Receptor Toll-like* 4 (TLR-4), que está presente em muitos tipos de células, incluindo macrófagos e células dendríticas (HOTCHKISS; MONNERET; PAYEN, 2013; MAGLIONE; SIMCHONI; CUNNINGHAM-RUNDLES, 2015). Inicialmente, o LPS é detectado por proteínas de ligação no plasma, que entrega a molécula formada ao CD14, possibilitando a ligação ao receptor TLR-4 junto a molécula acessória, denominada fator de diferenciação mielóide (MD-2) (BERTANI; RUIZ, 2018; COCHET, F.; PERI, F., 2017). Dessa forma, com a formação do complexo inflamatório (TLR-4/ MD-2/ LPS) é desencadeado o mecanismos de sinalização intracelular, promovendo a liberação de citocinas efetoras, como TNF-α, IL-1 e interleucina-1 (IL-6), além de aumentar o estresse oxidativo (COCHET, F.; PERI, F., 2017; HOTCHKISS; MONNERET; PAYEN, 2013).

2.2. Microbiota intestinal: composição e relação com processos fisiológico e patológicos

A microbiota intestinal humana é constituída por um vasto ecossistema de bactérias, composta por até 2000 espécies diferentes, 100 trilhões de bactérias com 150 vezes mais genes do que o genoma humano, com predomínio de bactérias anaeróbias (VIGGIANO; IANIRO; VANELLA; BIBBO *et al.*, 2015; XIE; HALEGOUA-DEMARZIO, 2019) e também outros microrganismos, tais como fungos e vírus (BIBBO; IANIRO; GIORGIO; SCALDAFERRI *et al.*, 2016). No entanto, a composição do trato intestinal humano é diferente em cada indivíduo, sendo influenciada por fatores genéticos, além de outros determinantes, como características individuais e ambientais, como o tipo de nascimento (parto normal ou cesariana), idade e hábitos alimentares (BARKO; MCMICHAEL; SWANSON; WILLIAMS, 2018; MILANI; DURANTI; BOTTACINI; CASEY *et al.*, 2017), o que resulta uma vasta variabilidade na formação desse microbioma (AL-NASIRY; AMBROSINO; SCHLAEPFER; MORRÉ *et al.*, 2020)

A microbiota do trato gastrointestinal humano é colonizada por quatro principais filos de bactérias, incluindo Firmicutes, Bacteroidetes, Actinobateria e Proteobacteria e menos frequentemente apresentam-se os filos Verrucomicrobia e Fusobacteria (LEY; HAMADY; LOZUPONE; TURNBAUGH *et al.*, 2008; XIE; HALEGOUA-DEMARZIO, 2019). Assim, a microbiota do trato gastrointestinal humano é composta por bactérias benéficas ou também chamadas de bactérias probióticas, e em menor proporção por bactérias nocivas (BELKAID; HARRISON, 2017; ZHOU; YUAN; ZHANG; GUO *et al.*, 2020). Como exemplo de probióticas, tem-se as Bifidobactérias e Lactobacilos (*Bacteróides* spp., *Bifidobacterium* spp., *Lactobacillus* spp., e para as nocivas encontram-se as Enterobacteriaceae e *Clostridium* spp. (BARKO; MCMICHAEL; SWANSON; WILLIAMS, 2018). São encontrados também na microbiota entérica a *Eubacterium* spp., *Fusonbacterium* spp., *Peptostreptococcus* spp., *Ruminococcus* (KIM; COVINGTON; PAMER, 2017).

A microbiota intestinal está envolvida em muitos processos biológicos fundamentais, incluindo regulação do desenvolvimento epitelial intestinal, modulação do metabolismo e estimulação da imunidade inata (AL-NASIRY; AMBROSINO; SCHLAEPFER; MORRÉ *et al.*, 2020; BELKAID; HARRISON, 2017). Além disso, a microbiota protege o corpo de patógenos externos através de colonização competitiva ou produção de agentes antimicrobianos como bacteriocinas, e ainda possui a capacidade de metabolizar substâncias químicas prejudiciais aos tecidos (FUNG; OLSON; HSIAO, 2017; WANG; HUANG; WANG; CAI *et al.*, 2019).

Por volta dos três anos de idade, a composição da microbiota intestinal torna-se semelhante ao de um adulto, e durante a fase de crescimento e desenvolvimento humano, o sistema imunológico pode precisar da interação da microbiota para desempenhar suas funções apropriadamente (WANG; HUANG; WANG; CAI *et al.*, 2019; ZHOU; YUAN; ZHANG; GUO *et al.*, 2020).

Os microrganismos comensais são necessários para a maturação do sistema imunológico, que precisa diferenciar as bactérias comensais das bactérias patogênicas (ZHOU; YUAN; ZHANG; GUO *et al.*, 2020). Os receptores *Toll Likes* (TLRs) da membrana das células epiteliais e linfóides do intestino delgado estão envolvidos nesse diferencial reconhecimento, sendo responsável pelo desenvolvimento normal do sistema imunológico da mucosa intestinal (JIAO; WU; HUNTINGTON; ZHANG, 2020). O papel dos TLRs é reconhecer diferentes tipos de padrões moleculares associados aos patógenos (PAMPs), como lipolissacarídeos, ácidos teicoico, dentre outros e acionar a resposta imune inata (BELKAID; HAND, 2014).

Em contribuição ainda, as células epiteliais intestinais produzem peptídeos antimicrobianos (α-defensinas, catelicidinas, lectinas do tipo C e lisozima), enquanto as células

caliciformes intestinais secretam glicoproteínas que formam uma camada de muco para evitar contato entre os microrganismos que compõe a microbiota e o tecido do hospedeiro (BELKAID; HARRISON, 2017).

A imunidade inata e adaptativa desempenham um papel importante na contenção e remoção de microrganismos patogênicos (XU; LIU; CAO; LI *et al.*, 2019). Esta interação deve-se ao tecido linfóide associado ao intestino (GALT), cujos componentes incluem principalmente as placas de Peyer e linfonodos mesentéricos, possuem células dendríticas, macrófagos e linfócitos T e B capazes de induzir respostas imunológicas (JIAO; WU; HUNTINGTON; ZHANG, 2020; STANISAVLJEVIĆ; LUKIĆ; MOMČILOVIĆ; MILJKOVIĆ *et al.*, 2016).

A composição e os metabólitos, como ácidos graxos de cadeia curta (AGCC), desse microambiente têm importantes contribuições na produção de anticorpos, modelando o repertório de células B, mantendo o equilíbrio entre as células Th17 e T reguladoras (Treg), e ainda regulando homeostase em diferentes subtipos de células T auxiliares (AL-NASIRY; AMBROSINO; SCHLAEPFER; MORRÉ *et al.*, 2020; RUFF; GREILING; KRIEGEL, 2020).

Os AGCCs, são resultantes da fermentação bacteriana de polissacarídeos indigeríveis, desempenham um importante papel antiflamatório (LEONG, 2018; ZHAO; ZHANG; DING; WU *et al.*, 2018). Estão também envolvidos em fornecer um substrato de energia aos colonócitos, promover reparo tecidual intestinal, além de auxiliar no tratamento de diversas doenças crônicas, como a DM2 (LEONG, 2018).

Deste modo, compreende-se que fatores como estilo de vida, dieta, envelhecimento e ingestão de antibióticos alteram a homeostase intestinal e propiciam o surgimento de muitas doenças, como reumatismo, diabetes tipo II, obesidade e doenças autoimunes (ZHOU; YUAN; ZHANG; GUO *et al.*, 2020).

2.3. Próbióticos no tratamento de condições clínicas associadas à endotoxemia

A homeostase da microbiota intestinal é um dos fatores essenciais para manter a saúde e promover proteção contra doenças no hospedeiro (RUFF; GREILING; KRIEGEL, 2020). A disbiose é definida como um desequilíbrio entre microrganismos saudáveis e promotores de doenças, é manifestada através de mudanças de diversidade e oscilação na abundância relativa de certos microrganismos (MARCHESI; ADAMS; FAVA; HERMES *et al.*, 2016; XIE; HALEGOUA-DEMARZIO, 2019). Desse modo, há um número crescente de estudos que revelam associação entre disbiose e síndrome metabólica, hipertensão, obesidade, diabetes tipo 2, doenças hepáticas gordurosas não alcoólicas (DHGNA), além de diversas

doenças inflamatórias intestinais (ALVAREZ-MERCADO; NAVARRO-OLIVEROS; ROBLES-SANCHEZ; PLAZA-DIAZ *et al.*, 2019; ROBLES-VERA; TORAL; DE LA VISITACION; SANCHEZ *et al.*, 2020; SHI; LV; FANG; WU *et al.*, 2017; XIE; HALEGOUA-DEMARZIO, 2019).

O termo probióticos refere-se a microrganismos vivos, não patogênicos, que possuem propriedades benéficas quando consumidos em doses adequadas, atuam na prevenção ou tratamento de determinadas doenças (SANCHEZ; DELGADO; BLANCO-MIGUEZ; LOURENCO *et al.*, 2017; SANDERS; MERENSTEIN; REID; GIBSON *et al.*, 2019). A colonização intestinal por probióticos, como *Lactobacillus* e *Bifidobacterium*, exerce uma barreira protetora ao organismo contra microrganismos patogênicos, de modo que uma disbiose na microbiota intestinal proporciona a evidenciação de bactérias entéricas Gram-negativas, como as Enterobacteriaceae, resultando na disseminação de infecções sistêmicas e distúrbios metabólicos (FEI; ZHAO, 2013). Nas seções a seguir são discutidos alguns aspectos da utilização de probióticos no tratamento de condições clínicas associadas ao estado endotoxêmico.

2.3.1. Probióticos e endotoxemia metabólica

A endotoxemia metabólica é caracterizada pela elevação dos níveis sanguíneos de LPS, independentemente da detecção de infecção bacteriana. Esta condição clínica é evidenciada pela propagação de doenças crônicas relacionadas à inflamação sistêmica, como obesidade, diabetes mellitus tipo 2, DHGNA, pancreatite, esclerose lateral amiotrófica, doenças cardiovasculares e doença de Alzheimer (FUKE, N.; NAGATA, N.; SUGANUMA, H.; OTA, T., 2019; MOLUDI; MALEKI; JAFARI-VAYGHYAN; VAGHEF-MEHRABANY *et al.*, 2020). A endotoxemia metabólica pode ser consequência de alta ingestão de açúcar que altera a homeostasia da microbiota, aumentando as propriedades pró-inflamatórias e diminuindo a capacidade de regular a integridade epitelial e a imunidade da mucosa (SATOKARI, 2020).

Evidências recentes demonstram que a disbiose da microbiota intestinal pode resultar no desenvolvimento de DHGNA relacionada à obesidade, além do aumento da permeabilidade intestinal favorecendo a translocação de microrganismos ou toxinas para circulação sanguínea (KOLODZIEJCZYK; ZHENG; SHIBOLET; ELINAV, 2019). De fato, pacientes com DHGNA apresentam disbiose intestinal associado a um aumento de Bacteroidetes e redução de Firmicutes o que resulta em esteatose hepática não alcóolica grave e inflamação (ALVAREZ-MERCADO; NAVARRO-OLIVEROS; ROBLES-SANCHEZ; PLAZA-DIAZ *et al.*, 2019). Atualmente, o LPS é considerado um dos principais atores na

patogênese e progressão da DHGNA, devido aos efeitos pró-inflamatórios e pró-fibrogênicos (CECCARELLI; PANERA; MINA; GNANI *et al.*, 2015).

Estudos experimentais e ensaios clínicos demonstram que a reestruturação da microbiota intestinal promove efeitos promissores na melhora da DHGNA. Camundongos com DHGNA tiveram redução signifitiva do peso e acúmulo de gordura visceral após administração oral de nove diferentes linhagens probióticas suplementada com galactooligossacarídeos (LIANG; LIANG; ZHANG; DENG *et al.*, 2019). Os mecanismos pelo qual isso acontece podem ser evidenciados por diversos fatores, como redução do acúmulo de gordura hepática, diminuição da endotoxemia, redução do estresse oxidativo, efeitos anti-inflamatórios através da modulação fator nuclear *kappa* B e TNF-α, além dos efeitos antifibróticos pela modificação da expressão do fator de crescimento (TGF-β) e do colágeno (XIE; HALEGOUA-DEMARZIO, 2019).

Outra condição clínica relacionada com a endotoxemia metabólica é a cirrose, onde a disbiose intestinal ocorre graças ao aumento de Enterobacteriaceae, causando complicações diversas como bacteremia e encefalopatia hepática, acompanhadas por crescimento excessivo de bactérias no intestino delgado e aumento da permeabilidade intestinal (AHLUWALIA; BETRAPALLY; HYLEMON; WHITE *et al.*, 2016; USAMI; MIYOSHI; YAMASHITA, 2015). Neste contexto patológico, ocorre aumento dos níveis circulantes de LPS e outras toxinas, caracterizando um quadro de endotoxemia metabólica (MANNISTO; FARKKILA; PUSSINEN; JULA *et al.*, 2019; WEIL; PAIS DE BARROS; MOUREY; LAHEURTE *et al.*, 2019). De fato, a endotoxemia derivada do intestino tem sido implicada no desenvolvimento de doença hepática crônica (GANDHI, 2020).

Certa ênfase tem sido dada no papel protetor dos probióticos na prevenção da cirrose através da modulação da microbiota intestinal (HONG; HAN; HONG; KIM *et al.*, 2019; PINZONE; CELESIA; DI ROSA; CACOPARDO *et al.*, 2012; RIVERA-FLORES; MORAN-VILLOTA; CERVANTES-BARRAGAN; LOPEZ-MACIAS *et al.*, 2020). Por exemplo, foi possível comprovar que a administração de probióticos (*Lactobacillus salivarius* LI01 ou *Pediococcus pentosaceus*) em um modelo experimental de cirrose induzida por tetracloreto de carbono (CCl4) teve a capacidade de reduzir endotoxina séricas por promover a integridade intestinal, redução de citocinas pró-inflamatórias, como TNF-α, IL-6 e IL-17A, além de propiciar o aumento de *Elusimicrobium* e *Prevotella,* e redução de *Escherichia coli* (SHI; LV; FANG; WU *et al.*, 2017).

2.3.2. Probióticos no tratamento da sepse e do choque séptico

A sepse é uma condição clínica com risco de vida, caracterizada por uma resposta desajustada do sistema imunológico ao nível sistêmico no intuito de combater uma infecção (FITZPATRICK; LAMBDEN; MACIAS; PUTHUCHEARY *et al.*, 2020; KOUTROULIS; BATABYAL; MCNAMARA; LEDDA *et al.*, 2019; VAN DER POLL; VAN DE VEERDONK; SCICLUNA; NETEA, 2017). Altas concentrações sanguíneas de LPS (como parte da bactéria ou na forma secretada) pode levar ao estabelecimento do choque séptico, devido à ativação do receptor TLR-4 (YAN; LIANG; ZHOU; HUANG *et al.*, 2020). Devido à disfunção de múltiplos órgãos, o choque séptico é um problema sério para o sistema de saúde em todo o mundo, devido às altas taxas de morbimortalidade que fazem da sepse uma das dez principais causas de mortes (FLEISCHMANN; SCHERAG; ADHIKARI; HARTOG *et al.*, 2016; NEIVERTH; PRIM; FRANCK; NISIHARA, 2020; QUINTANO NEIRA; HAMACHER; JAPIASSU, 2018).

Vários fatores estão relacionados ao alto número de casos de sepse que incluem a disseminação de genes envolvidos na virulência e resistência microbiana e a (re) emergência de alguns patógenos (LIN; MCGINLEY; DRYSDALE; POLLARD, 2018; MONTEIRO; PINTO; MONTEIRO; FERREIRA *et al.*, 2019; RUIZ-GAITAN; MORET; TASIAS-PITARCH; ALEIXANDRE-LOPEZ *et al.*, 2018). Além disso, aspectos relacionados ao indivíduo (como idade, doenças metabólicas e outras comorbidades) podem influenciar o resultado da sepse (NEIVERTH; PRIM; FRANCK; NISIHARA, 2020; SHANKAR-HARI; SAHA; WILSON; PRESCOTT *et al.*, 2020). Devido à complexidade da fisiopatologia da sepse, o desenvolvimento de terapias eficazes ainda é um grande desafio (CAVAILLON; SINGER; SKIRECKI, 2020; VAN DER POLL; VAN DE VEERDONK; SCICLUNA; NETEA, 2017).

Algumas evidências mostraram fortes efeitos imunomoduladores induzidos por bactérias probióticas (particularmente certas cepas de *Lactobacillus* sp.) em modelos experimentais de distúrbios inflamatórios (ABDO; LECUREUX; LAVOY; EKLUND *et al.*, 2019; SIRESWAR; BISWAS; DEY, 2020; VAREILLE-DELARBRE; MIQUEL; GARCIN; BERTRAN *et al.*, 2019). Esses resultados advogam seu possível uso no tratamento da sepse (AVILA; MICHELS; VUOLO; BILESIMO *et al.*, 2020; HARO; MONACO; MEDINA, 2018). Essas bactérias podem modular a resposta do hospedeiro devido à estimulação direta das células imunológicas e / ou modificação da microbiota intestinal (AVILA; MICHELS; VUOLO; BILESIMO *et al.*, 2020; MAO; QI; CUI; DOU *et al.*, 2020; MAZZEO; LUONGO;

SASHIHARA; ROSSI *et al.*, 2020; NATION; DUNNE; JOSEPH; MENSAH *et al.*, 2017; NOGACKA; ODDI; SALAZAR; REINHEIMER *et al.*, 2019) PMID: 32027752).

Em modelo animal foi possível observar que o tratamento com *Lacticaseibacillus rhamnosus* L34 reduziu o número de bactéria patogênicas fecais, além da produção de citocinas pro-inflamatórias como IL-6 por células epiteliais do cólon, após indução de sepse e administração de antibiótico via oral (PANPETCH; CHANCHAROENTHANA; BOOTDEE; NILGATE *et al.*, 2018). Em outro estudo, a administração oral de *Lactobacillus casei* CERELA (CRL) 431 após indução de sepse induzida por LPS proporcionou redução de TNF-α e IL-6, e ainda redução da ativação do sistema de coagulação (HARO; MÓNACO; MEDINA, 2018).

2.3.3. Probióticos como agentes antioxidantes

Uma clássica consequência do reconhecimento do LPS pelo sistema imunológico é a liberação de espécies reativas de oxigênio (EROs) e de nitrogênio (ERNs) como o superóxido e óxido nítrico (BERTANI; RUIZ, 2018; CHANG; YEH; HO; LIU *et al.*, 2019). É válido ressaltar que estas espécies reativas têm funções imunológicas e fisiológicas importantes, são geradas durante o metabolismo oxidativo normal do indivíduo (BALMUS; CIOBICA; ANTIOCH; DOBRIN *et al.*, 2016). Além disso, o hospedeiro possui um sistema de defesa antioxidante responsável pela eliminação destas espécies, promovendo a proteção do organismo ao estresse oxidativo gerado (BALMUS; CIOBICA; ANTIOCH; DOBRIN *et al.*, 2016; TAN; NORHAIZAN; LIEW, 2018). O sistema antioxidante incluí as enzimas superóxido dismutase, catalase, heme oxigenase-1 e glutationa-S-transferases (KLENIEWSKA; PAWLICZAK, 2017).

O estresse oxidativo desempenha um papel importante na fisiopatologia das manifestações sistêmicas da endotoxemia incluindo disfunções hepática, cerebral, cardíaca, pulmonares e no trato gastrointestinal (CHANG; YEH; HO; LIU *et al.*, 2019; PRONIEWSKI; KIJ; SITEK; KELLEY *et al.*, 2019; TAN; WAN; SUN; ZHANG *et al.*, 2020). Isto ocorre porque o estado endotoxêmico leva à indução danos oxidativos em macromoléculas (proteínas, lipídios, carboidratos e DNA), prejudicando o metabolismo celular e tecidual (YORULMAZ; OZKOK; KAPTAN; ATES *et al.*, 2018). Desta maneira a inibição do estresse oxidativo é considerado um alvo potencial para reduzir as consequências deletérias da endotoxemia sistêmica (CHANG; YEH; HO; LIU *et al.*, 2019).

Algumas cepas probióticas têm demonstrado potencial atividade antioxidante em modelos *in vivo* e *in vitro* (KOBATAKE; NAKAGAWA; SEKI; MIYAZAKI, 2017;

NAKAGAWA; SHIOZAKI; KOBATAKE; HOSOYA *et al.*, 2016; WU; WANG; XU; TANG *et al.*, 2019). Os mecanismos pelos quais isso acontece estão associados a produção e excreção de metabólitos antioxidantes, modulação da atividade antioxidante, redução da atividade enzimática de espécies reativas de oxigênio, além da indução da autofagia de células que sofreram danos (KOBATAKE; NAKAGAWA; SEKI; MIYAZAKI, 2017; PEREIRA; FEITOSA; ABREU; LEMOS *et al.*, 2017; WU; WANG; XU; TANG *et al.*, 2019).

Outros dados demonstram que o tratamento de camundongos hipertenso com probióticos, como *Bifidobacterium breve* CECT7263 e *Limosilactobacillus fermentum* CECT5716 demonstrou aumento de bactérias produtoras de butirato na microbiota intestinal, promovendo o relaxamento endotelial e redução da produção de espécies reativas de oxigênio, ao impedir o aumento na interação sistêmica de LPS e TLR-4, além do aumento da população de células Treg no endotélio, obtendo como resultado final a redução da pressão arterial (ALVAREZ-MERCADO; NAVARRO-OLIVEROS; ROBLES-SANCHEZ; PLAZA-DIAZ *et al.*, 2019; ROBLES-VERA; TORAL; DE LA VISITACION; SANCHEZ *et al.*, 2020).

Visto que a hiperglicemia crônica e disbiose intestinal são consideradas fontes importantes na produção de ROS, o uso de substâncias com propriedades prebióticas como a dextrina, um polímero de glicose derivado principalmente do milho e trigo, em indivíduos com Diabetes Mellitus Tipo 2 (DM2) foi capaz de promover uma melhora no perfil lipídico e na redução de níveis séricos de proteína C reativa (PCR) e LPS (FARHANGI; DEHGHAN; NAMAZI, 2019) .

Portanto, parece que a redução do estresse oxidativo, endotoxemia metabólica e respostas inflamatórias, juntamente com o equilíbrio da microbiota intestinal podem contribuir para prevenção de possíveis complicações das doenças crônicas não transmissíveis (CHANG; YEH; HO; LIU *et al.*, 2019; ROBLES-VERA; TORAL; DE LA VISITACIÓN; SÁNCHEZ *et al.*, 2020).

2.4. *Lacticaseibacillus rhamnosus* ATCC 9595 uma cepa com potencial probiótico

Lacticaseibacillus rhamnosus, anteriormente denominado de *Lactobacillus rhamnosus* é um microrganismo Gram-positivo do grupo de bactérias ácido-láticas, caracterizado como anaeróbica facultativa e heterofermentativa, que produz ácido láctico L(+), acético, fórmico e etanol, em condições de anaerobiose (BERNARDO; COELHO; SASS; CONTIERO, 2016). Esta é uma das espécies de probióticos mais estudadas por apresentar cepas capazes de colonizar e proteger o intestino delgado (PACE; PACE; QUARTARONE, 2015), prevenir diarreia (de etiologia bacteriana ou viral) e outras doenças infecciosas (FIGUEROA-

GONZÁLEZ; CRUZ-GUERRERO; QUIJANO, 2011; HE; ZENG; PUTHIYAKUNNON; ZENG *et al.*, 2017; LIU; WU; WANG; FU *et al.*, 2016).

Diversos estudos demonstraram que a ação anti-infecciosa de *L. rhamnosus* está relacionado com a modulação do sistema imunológico do hospedeiro, além de conferirem proteção às mucosas através da adesão às membranas, inibindo infecções fúngicas e bacterianas (COSTABILE; BERGILLOS-MECA; RASINKANGAS; KORPELA *et al.*, 2017; SALIGANTI; KAPILA; KAPILA, 2016). Adicionalmente, linhagens de *L. rhamnosus* são capazes aliviar reações de hipersensibilidade e a inflamação intestinal (SALIGANTI; KAPILA; SHARMA; KAPILA, 2015; YAN; LIU; CAO; MOORE *et al.*, 2017) , sendo também indicadas como coadjuvante em casos de neoplasias, eczema, diarreia, intolerância à lactose, inflamação intestinal e infecções dos tratos vaginal e urinário (VAJRO; MANDATO; LICENZIATI; FRANZESE *et al.*, 2011).

L. rhamnosus é muito utilizado na produção de alimentos probióticos, como iorgutes, bebida de soja e sucos fermentados (AGUILAR-TOALÁ; GARCIA-VARELA; GARCIA; MATA-HARO *et al.*, 2018; KUO; WANG; LU; HU *et al.*, 2013; TIAN; CHI; WANG; LIU *et al.*, 2015). Deste modo, inúmeras linhagens de *L. rhamnosus* têm sido prospectadas quanto seu potencial probiótico em diferentes modelos experimentais. Um exemplo é a cepa *Lacticaseibacillus rhamnosus* ATCC 9595.

Foi possível observar que das diversas linhagens de lactobacilos utilizadas para verificar se os compostos ativos de *Lactobacillus spp* apresentavam atividade antifúngica e antivirulência, destacou-se o *L. rhamnosus* ATCC 9595, demonstrando que a produção de biossurfactantes indiziu redução da adesão dos isolados vaginais de *Candida albicans,* promovendo a interrupção da formação de biofilme (ITAPARY DOS SANTOS; RAMOS FRANCA; DUARTE LIMA CAMPOS; QUARESMA BOMFIM *et al.*, 2019).

Ao ser investigado a ação probiótica de *L. rhamnosus* ATCC 9595 no modelo de larvas *Galleria mellonella* infectada com *Candida albicans* foi verificado aumento no tempo de sobrevivência das larvas ao inibir fatores de virulência fúngica e modulação do sistema imunológico que possivelmente está associado ao recrutamento de hemócitos para hemolinfa (RIBEIRO; DE BARROS; ROSSONI; JUNQUEIRA *et al.*, 2017).

Deste modo, a produção de substâncias como ácidos orgânicos, peróxido de hidrogênio, bacteriocinas, moléculas antimicrobianas, além de biossurfactantes por cepas probióticas, todos os quais podem impedir o crescimento de possíveis patógenos (GASPAR; DONDERS; PALMEIRA-DE-OLIVEIRA; QUEIROZ *et al.*, 2018; TAHMOURESPOUR; KASRA-KERMANSHAHI; SALEHI, 2019).

Além do mais, exopolissacarídeos (EPS) produzidos por *L. rhamnosus* ATCC 9595 exibiu atividade imunossupressora ao ser testado em macrófagos peritoneais de camundongos estimulados com lipopolissacarídeo (LPS), induzindo a produção de altos níveis de IL-10 e redução dos níveis de TNF-α (BLEAU; MONGES; RASHIDAN; LAVERDURE *et al.*, 2010)

2.5. Bacuri e Cupuaçu: alternativas para o desenvolvimento de produto probióticos

A fermentação lática de produtos alimentícios é uma alternativa interessante e bastante aplicada para a obtenção de produtos diferenciados, com vários benefícios à saúde que se agregam ao valor positivo daqueles da versão não fermentada (DIMIDI; COX; ROSSI; WHELAN, 2019; FARAG; EL HAWARY; ELMASSRY, 2019; SLATTERY; COTTER; O'TOOLE, 2019). Probióticos são comumente encontrados em produtos lácteos, como leites, iogurtes e queijos (MIYAZIMA; ISHIKAWA; MAYER; SAAD *et al.*, 2017; YOON; CHA; HONG; KIM *et al.*, 2019; YUKI; FURUTANI; MIZOTA; WAKITA *et al.*, 2019). Entretanto, apesar deste tipo de fermentação estar muito mais frequentemente associada ao leite, ela também pode ser realizada a partir de outros substratos, como as polpas de frutas, desde que possuam açúcares fermentáveis em suas composições (MANTZOURANI; KAZAKOS; TERPOU; ALEXOPOULOS *et al.*, 2018; MANTZOURANI; NOUSKA; TERPOU; ALEXOPOULOS *et al.*, 2018; NGUYEN; BUJNA; FEKETE; TRAN *et al.*, 2019).

Os sucos de frutas podem ser excelentes veículos para o desenvolvimento de produtos probióticos devido às suas propriedades nutricionais e à presença de compostos bioativos (vitaminas, ácidos fenólicos, flavonóides e outros compostos antioxidantes), resultando em produtos com características sensoriais próprias (LIU; CHENG; LIU; MA *et al.*, 2019; LU; TAN; CHEN; LIU, 2018; RICCI; CIRLINI; MAOLONI; DEL RIO *et al.*, 2019). O uso de sucos de frutas contendo probióticos também oferece oportunidades para indivíduos com condições específicas (intolerância à lactose, alergia a componentes do leite e vegetarianos) se beneficiarem do consumo dessas bactérias (BANCALARI; CASTELLONE; BOTTARI; GATTI, 2020; MANTZOURANI; KAZAKOS; TERPOU; ALEXOPOULOS *et al.*, 2018).

O Brasil é um dos países que tem o maior repertório de frutas em todo o mundo, constituindo uma abrangente quantidade de frutas tropicais e exóticas com aromas e sabores únicos (TEIXEIRA; MELO; BATISTA; PAULA-SOUZA *et al.*, 2019). São exemplos os frutos das espécies *Platonia insignis* (Clusiaceae) e *Theobroma grandiflorum* (Malvaceae), conhecidos popularmente como bacuri e cupuaçu, respectivamente. Estes frutos são típicos da região da Amazônia Legal, sendo também encontrados em outros biomas brasileiros, como o

cerrado. A seguir são apresentadas algumas características que tornam estas frutas candidatas na produção.

2.5.1. Bacuri

O bacurizeiro tem origem Amazônica, mas também é cultivada no Cerrado brasileiro (nos estados do Pará, Maranhão e Piauí), desempenhando um importante papel econômico na região Norte-Nordeste do Brasil (CARVALHO; NASCIMENTO; DO, 2018; PONTES; MOURA; MOURA; RODRIGUES *et al.*, 2017).

O bacuri (*Platonia insignis* Mart.) é um fruto redondo, com casca grossa, e de cor amarelo-citrina, contendo polpa viscosa, muito saborosa, sendo utilizada em doces, licores e sorvetes (Figura 1) (CARVALHO; MÜLLER, 2007; SILVA; FIGUEIREDO; BRITO; MAIA *et al.*, 2010). Além disso, tem sido demonstrado o potencial antioxidante e inibidor da α-glucosidase da polpa do fruto (DE FREITAS; ARAUJO; SOARES; NUNOMURA *et al.*, 2018). De fato, a abundância de compostos bioativos (como ácido cítrico, ácido p-cumárico e terpenos) presentes no bacuri tem a capacidade de potencializar os efeitos benéficos de bactérias probióticas (DE FREITAS; ARAUJO; SOARES; NUNOMURA *et al.*, 2018; UEKANE; NICOLOTTI; GRIGLIONE; BIZZO *et al.*, 2017).

Figura 1: Polpa de *Platonia insignis* Mart.

Fonte: https://www.embrapa.br/busca-de-imagens/-/midia/3834001/fruto-do-bacurizeiro

Adicionalmente, os açúcares presentes na fruta (glicose, frutose e sacarose), vitaminas C, E e metais como (Na, K, Ca, Mg, P, Fe, Zn e Cu), superiores aos observados em outras frutas Amazônicas, como araçá-boi e cupuaçu, podem proporcionar o crescimento do probiótico (MARIA DO SOCORRO; ALVES; DE BRITO; PÉREZ-JIMÉNEZ *et al.*, 2010).

Deste modo, o suco do bacuri é um veículo interessante para o desenvolvimento de produtos fermentados.

Tabela 1. Compostos funcionais e propriedades funcionais identificados na polpa do Bacuri.

Tecido	Composto funcionais	Propriedades funcionais	Referência
Polpa	Aminoácidos: ácido aspártico, ácido glutâmico Ácidos graxos: ácido oléico, ácido palmítico	Antipilético; neuroproteção	(ROGEZ; BUXANT; MIGNOLET; SOUZA *et al.*, 2004); (XIANG; JIANG, 2013); (SONG; KIM; LEE; LEE *et al.*, 2019)
Polpa	Compostos fenólicos (flavonoides)	Antioxidante, antimicrobiano	(DE FREITAS; ARAUJO; SOARES; NUNOMURA *et al.*, 2018)
Polpa	Ácido cítrico	Antiflamatório e a antioxidante	(DE FREITAS; ARAUJO; SOARES; NUNOMURA *et al.*, 2018); (ABDEL-SALAM; YOUNESS; MOHAMMED; MORSY *et al.*, 2014)

2.5.2. Cupuaçu

O cupuaçu é uma das 22 espécies do gênero *Theobroma,* pertencente à família Sterculiaceae (QUIJANO; PINO, 2007). O cupuaçuzeiro (*Theobroma grandiflorum*) é uma planta encontrada naturalmente nas florestas tropicais brasileiras, especialmente nos estados do Amazonas, Pará, Maranhão, Rondônia e Acre (PUGLIESE; TOMAS-BARBERAN; TRUCHADO; GENOVESE, 2013). Seu cultivo é distribuído por toda a Amazônia, sendo uma das frutas mais populares da região devido as agradáveis características de aroma e sabor da polpa (AVILA-SOSA; MONTERO-RODRÍGUEZ; AGUILAR-ALONSO; VERA-LÓPEZ *et al.*, 2019).

Os frutos possuem casca dura e lisa de cor castanho-escuro (Figura 2), mas facilmente quebrável, onde as sementes ficam envolvidas pela polpa, que é branca, ácida e

fibrosa, com um aroma intenso e agradável, além de alto valor nutricional, o que o torna muito apreciado (DE HOLANDA; CLÍMACO; HUNALDO; GOMES *et al.*, 2020). Pode ser utilizado *in natura* ou em preparação industrial para a produção de sucos, sorvetes, picolés, geleias, bombons, doces, sendo uma importante fonte de vitaminas e minerais (PUGLIESE; TOMAS-BARBERAN; TRUCHADO; GENOVESE, 2013).

Figura 2: Polpa de *Theobroma grandiflorum*.

Fonte: https://www.embrapa.br/busca-de-imagens/-/midia/5041001/cupuacu-aberto-polpa

O cupuaçu possui propriedades antioxidantes como as vitaminas C (ácido ascórbico) e E (tocoferóis), flavonoides, antocianinas e polifenóis, além de sacarídeos (glicose, frutose e sacarose) e minerais (como Na, K, Ca, Mg, P, Fe, Zn e Cu) (DE HOLANDA; CLÍMACO; HUNALDO; GOMES *et al.*, 2020; DE MORAES BARROS; GARCÍA-VILLALBA; TOMÁS-BARBERÁN; GENOVESE, 2016; PUGLIESE; TOMAS-BARBERAN; TRUCHADO; GENOVESE, 2013).

Tabela 2. Compostos funcionais e propriedades funcionais identificados na polpa do Cupuaçu

Tipo de material	Composto funcionais	Propriedades funcionais	Referências
Polpa	Flavonas conjugadas (clovamida, epicatequina)	Neuroproteção; atividade antitumoral;	(FALLARINI; MIGLIO; PAOLETTI; MINASSI *et al.*, 2009); (DE MORAES BARROS; GARCÍA-VILLALBA; TOMÁS-BARBERÁN; GENOVESE, 2016); (TAKANASHI; SUDA; MATSUMOTO; ISHIHARA *et al.*, 2017)
Vinho preparado da polpa	Ácidos: Ácido butírico, Ácido hexanóico	Anti-inflamatório	(KESHARI; BALASUBRAMANIAM; MYAGMARDOLOONJIN; HERR *et al.*, 2019); (DUARTE; DIAS; OLIVEIRA; TEIXEIRA *et al.*, 2010)
Licor preparado da polpa	Compostos fenólicos: Proantocianidina	Antioxidante, antiflamatório, anti-tumoral; redução da hipertrigliceridemia.	(DE OLIVEIRA; GENOVESE, 2013; LAI; XIAN; XIONG; YANG *et al.*, 2018)

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LISTA DE PUBLICAÇÕES

CAPÍTULO DE LIVRO 1: Avaliação das atividades antimicrobiana e antioxidante de extratos obtidos das frutas *theobroma grandiflorum* e *Mauritia flexuosa.*

ARTIGO 1: Short-term intake of *Theobroma grandiflorum* juice fermented with *Lacticaseibacillus rhamnosus* ATCC 9595 amended the outcome of endotoxemia induced by lipopolysaccharide

ARTIGO 2: Avaliação do crescimento e viabilidade de bactérias probióticas (*Limosilactobacillus fermentum* ATCC 23271 e *Lacticaseibacillus rhamnosus* ATCC 9595) em suco de bacuri (*Platonia insignis*)

CAPÍTULO 7

AVALIAÇÃO DAS ATIVIDADES ANTIMICROBIANA E ANTIOXIDANTE DE EXTRATOS OBTIDOS DAS FRUTAS *Theobroma grandiflorum* E *Mauritia flexuosa*

George Barros Chaves
Universidade CEUMA, Curso de Nutrição, São Luís – Maranhão.

Gabrielle Damasceno Evangelista Costa
Universidade CEUMA, Curso de Nutrição, São Luís – Maranhão.

Maria Clara Caldas Costa
Universidade CEUMA, Curso de Nutrição, São Luís – Maranhão.

Yasmim Costa Mendes
Universidade CEUMA, Curso de Nutrição, São Luís – Maranhão.

Gabrielle Pereira Mesquita
Universidade CEUMA, Curso de Nutrição, São Luís – Maranhão.

Lívia Muritiba Pereira de Lima Coimbra
Universidade CEUMA, Curso de Nutrição, São Luís – Maranhão.

Luís Cláudio Nascimento da Silva
Universidade CEUMA, Curso de Biomedicina e Programa de Pós Graduação em Biodiversidade e Biotecnologia (REDE BIONORTE), São Luís – Maranhão.

Adrielle Zagmignan
Universidade CEUMA, Curso de Nutrição e Programa de Pós Graduação em Biodiversidade e Biotecnologia (REDE BIONORTE), São Luís – Maranhão.

RESUMO: O cenário das epidemias causadas pelas doenças transmitidas por alimentos e aumento de doenças crônicas faz necessárias investigações a respeito de novas substâncias com propriedades antimicrobianas e antioxidantes. O objetivo do estudo foi avaliar as atividades antimicrobiana, antioxidante, citotóxica e mutagênica de extratos obtidos das frutas *Theobroma grandiflorum* (cupuaçu) e *Mauritia flexuosa* (buriti). Foi realizado um estudo experimental, no Laboratório de Pesquisa em Microbiologia e Imunologia na Universidade Ceuma. As frutas foram coletadas em Fevereiro de 2017, passando pelo processo de extração da polpa e preparo das frações Hexânica, Clorofórmica, Acetato de Etila e Metanólica para realização dos ensaios de concentração inibitória mínima com as cepas *Salmonellas* ATCC 14028 e *Staphylococcus aureus* ATCC 6538, além dos ensaios DPPH (2,2-difenil-1-picrilhidrazilo; Sigma-Aldrich), hemólise e mutagenicidade. Nos ensaios de capacidade inibitória mínima os extratos não apresentaram ações antimicrobianas, no ensaio DPPH, as frações acetato de etila e metanólica do cupuaçu mostraram atividade antioxidante de 60%, enquanto que o buriti nas mesmas frações apresentou 45% e 50% respectivamente, sem grandes consequências lesivas às hemácias. As frações testadas para os diferentes extratos a 100 mg/mL não apresentaram ação mutagênica. Nos ensaios de capacidade inibitória mínima os extratos

ARTIGO 1

Short-term intake of *Theobroma grandiflorum* juice fermented with *Lactobacillus rhamnosus* ATCC 9595 amended the outcome of endotoxemia induced by lipopolysaccharide

Abstract:

Endotoxemia is a life-threatening clinical condition, characterized by an impaired systemic response that causes multiple organ dysfunction and represents a serious problem for care with the health system worldwide. Some evidence has shown strong immunomodulatory effects induced by probiotic bacteria in experimental models of inflammatory disorders. It is associated with the use of fruits as a vehicle for the growth of these probiotic bacteria. In this context, the intake of fermented juice of *Lactobacillus rhamnosus* (*L. rhamnosus*) in mice submitted to lipopolysaccharide-induced endotoxemia (LPS). After optimizing the best cultivation condition for *L. rhamnosus* in different pulp concentrations in cupuaçu juice, in addition to better production of lactic acid, the C57BL / 6 mice (n = 12 / group) were randomly allocated to experimental groups that received doses oral (100 µL / mouse) of phosphate buffered saline (PBS), unfermented cupuaçu juice or cupuaçu juice fermented by *L. rhamnosus* (resulting 10^8 CFU / mL) for five days. Each animal was placed in an individual cage and received an intraperitoneal injection of LPS and was sacrificed after 6 h and 120 h after inoculation with LPS. The severity of endotoxemia was also daily evaluated. Every day, body weight and temperature were recorded and compared with data obtained before inoculation of LPS (baseline). The organs of each animal were weighed and measured, in addition to the puncture of cardiac blood to measure total and differential cells. The main results obtained suggest that the administration of *L. rhamnosus*-fermented juice reduced the severity of LPS-mediated endotoxemia, reduced weight loss, amended the migration of cells to peritoneal cavity and reduced the increase of cells in blood. In conclusion, our data show that early therapy of fermented cupuaçu juice with *Lactobacillus rhamnosus* ATCC 9595 can reduce systemic inflammation in an experimental model of sepsis in mice.

1. Introduction

Lipopolysaccharide (LPS) is a structural component of the outer membrane of Gram-negative bacteria [1] that can be recognized by the Toll-like Receptor 4 (TLR-4) [2,3]. Increased levels of LPS in the bloodstream are classified as endotoxemia, which may result from bacteremia and/or metabolic dysfunctions [4]. Metabolic endotoxemia is closely related to changes in intestinal microbiota homeostasis, as consequence of obesity, liver damage or other inflammatory-related chronic conditions [4-6].

Regardless of its causes, the endotoxemia is a life-threatening clinical condition characterized by an impaired systemic response that provokes the dysfunction of multiple organs and represents a is a serious issue for health system care worldwide [7,8]. Due the complexity of endotoxin shock physiopathology, the development of effective therapy still a huge challenge [7,9].

Some evidences have shown the strong immunomodulatory effects induced by probiotic bacteria (particularly certain *Lactobacillus* sp. strains) in experimental models of inflammatory disorders [10-12]. These results advocate for their possible alternative use in the treatment of the clinical conditions related to endotoxemia [13,14]. These bacteria may modulate host response due direct stimulation of immune cells and/or modification of gut microbiota [13,15-18].

Lactobacilli are lactic acid bacteria that are normal inhabitants of the human gut and they have been also consumed as food for a long time [19]. However, the majority of products containing *Lactobacillus* are derived from milk, imposing an obstacle for some individuals (such as those with lactose intolerance, allergy of milk protein and vegans) pointing for the importance of plant derived material for obtention of new probiotic products [20]. In this sense, the use of juice as vehicles for these probiotic bacteria are emerging a suitable alternative [21,22]. Furthermore, the available of various bioactive compounds in juices may act in synergy with the *Lactobacillus* to promote the beneficial effects for the consumers [23,24].

The juice from *Theobroma grandiflorum* (Cupuassu or Cupuaçu) is very appreciate in Brazil *in nature* or in industrial preparation for the production of juices, ice creams, popsicles, jams, chocolates, sweets, being an important source of vitamins and minerals [25] and has antioxidant properties such as vitamins C (ascorbic acid) and E (tocopherols), flavonoids, anthocyanins and polyphenols, in addition to saccharides (glucose, fructose and sucrose) and minerals (such as Na, K, Ca, Mg, P, Fe, Zn and Cu) [25-27]. This work analyzed the growth of *Lactobacillus rhamnosus* ATCC 9595 in

cupuaçu juicy and the production of lactic acid. Then, we evaluated the effects of short-term intake of *L. rhamnosus*-fermented juice in mice submitted to endotoxemia induced by LPS.

2. Methods

2.1. Origin and maintenance of probiotic strains

L. rhamnosus ATCC 9595 and was kindly provided by Dr. Valério Monteiro Neto's Bacterial collection maintained in *Universidade Ceuma*. The strain is kept refrigerated at -80 °C. For each experiment, aliquots were activated in MRS broth (De Man, Rogosa and Sharpe). The analyzes to determine the proximate composition of *T. grandiflorum* were carried out in triplicate [28]. The Nifext fraction (carbohydrates) was obtained by calculating the difference from the other fractions analyzed. The composition of fatty acids was determined by the Soxhlet method, as well as the determination of moisture and ash were performed as described in the physical-chemical methods for food analysis [29] (Table 1).

2.2. Fermentation

The fruits of *T. grandiflorum* were collected in Açailandia (Maranhão, Brazil). A sample of the plant (branch with leaf, flower and fruit) was sent to the Herbarium "Ático Seabra" of the Federal University of Maranhão (UFMA) for identification. The fruit pulp was manually removed and stored at -20ºC. In the initial fermentation assays, the pulp sample (30 g) was dissolved in 250 mL of distilled water to reach a concentration of 120 mg/mL. The pH was adjusted to 6.0 before sterilization. In parallel, a pre-inoculum was prepared in MRS broth. Probiotic cells were grown at 37 ° C under agitation (120 rpm). After 24 h, 1 mL aliquots of each bacterial suspension [optical density at 600 nm (OD_{600nm}) = 1.0] were inoculated in juice or MRS broth. The cultures were incubated with shaking at 120 rpm for 48 hours.

The quantification of bacterial growth was performed by plating on MRS agar. Serial dilutions were made in PBS solution after each determined period (0, 7, 14, 21 and 28 days of refrigeration). Then, the Petri dishes were incubated for 48 hours at 37 ° C. The number of bacteria was determined by counting colony-forming units (CFU) and expressed in CFU/mL.

2.3. Optimization of cultivation conditions

The optimization of cultivation conditions was carried out through a Central Rotational Composite Design (DCCR). The two variables chosen were the inoculum concentration (x_1) and the pulp concentration (x_2), with a total of 10 experiments (Table 2). After each test, the pH values and the microbial population were analyzed to determine the relationship between bacterial growth and pH (G/pH), as well as better production of lactic acid [30].

2.3.1. Quantification of lactic acid content

The quantification of lactic acid in the fermentative liquid was performed using a Shimadzu high-performance liquid chromatograph, equipped with a quaternary pump r coupled to a degassing system (DGU-20A5r). The system contains an oven to control the column temperature (set at 28 ° C) and an automatic injector (20 µL injection) with a diode array detector (SPD-M20A; range 190-800 nm). An ion exchange column (300 mm x 7.8 mm x 9 µm; Aminex® HPX-87H, Bio-Rad, USA) was used. The elution was carried out isocratically with a mobile phase composed of 5 mM H_2SO_4 and with a flow of 0.6 mL/min. The software used was LC-Solutions manufactured by Shimadzu Corporation (Kyoto, Japan) [30]. For each test, the concentration of lactic acid in an unfermented juice containing the same concentration of pulp (unfermented controls) was also detected. The production of lactic acid (g/L) was determined by the difference between the concentration of lactic acid in each fermented liquid and its respective unfermented control. The fermentative conditions with best results were selected for *in vivo* assays.

2.4. Animal Experimentation

2.4.1. Animals

This study used male C57BL/6 mice aged 6–8 weeks and weighing 20–25 g. The animals were housed in plastic cages at room temperature (23 ± 1°C) and submitted to 12 h light-dark cycle. They received balanced laboratory food and water ad libitum. All experimental procedures were conducted following the laboratory animal care standards

of the CEUMA University Animal Experimentation and Use Committee (approval N° 68/17).

2.4.2. Short-term administration of *L. rhamnosus*-fermented and unfermented cupuaçu juice

The animals were allocated in experimental groups (*n*=12/group) that received oral doses (100 µL/mouse) of phosphate-buffered saline (PBS) (controls groups/*n*=20), unfermented cupuaçu juice or *L. rhamnosus*-fermented cupuaçu juice (resulting 10^8 CFU/mL).

Another groups (control) received 100 µL of. The oral administration of each sample was performed for 5 days.

2.4.3. Induction and evaluation of LPS-mediated endotoxemia.

Each animal was placed in individual cage in received an intraperitoneal injection of LPS (10 mg/kg in saline; obtained from *E. coli* serotype O111:B4; Sigma-Aldrich) [31]. Every day the body weight and temperature were recorded and compared with the data obtained prior the LPS inoculation (baseline). The severity of endotoxemia was also daily evaluated using a score as reported by [32] which is based on the observation on grooming behavior, mobility, presence of piloerection and weeping eyes. The animals (*n*=6/group) were euthanized after 6 h and 120 h following LPS inoculation using lethal doses of 80 mg/kg ketamine hydrochloride and 10 mg/kg xylazine hydrochloride.

2.4.4. Determination of Cell population in the blood and Peritoneal Cavity

The blood was collected by cardiac puncture using tubes containing EDTA and aliquots were reserved for analysis of total and differential cell population. Following, the samples were centrifuge (2000 rpm, 4 °C, 20 min) and the plasma samples were obtained and stored at -80 °C. In parallel, the mice were submitted to laparotomy followed by the introduction of 3 mL of EDTA (1 mM in PBS) into the abdominal cavity. The peritoneal lavage fluids (PELF) were transferred to a tube and stored at -80 °C. Total and differential measurement of cell population were also performed in PELF samples.

The leukocytes present in each sample were counted in a Neubauer chamber under microscopy (×10 objective) after appropriate dilution in *Türk* solution. The differential determination of polymorphonuclear (PMN) and mononuclear (MN) leukocytes were performed using a 100 µm hanging drop of sample obtained by cytocentrifugation at 600 rpm for 10 min. The slides were Giemsa stained, and the 100 cells were counted by optical microscopy at 1000 × using an oil immersion objective.

2.5. Statistical Analysis

Data were presented as means ± standard variation (SD) or percentages. The data were analyzed using the softwares GraphPad Prism® (version 7.0) or *Statistica*®. The normality of distributions was determined by the Shapiro-Wilk test, and the differences between groups were evaluated by analysis of variance (ANOVA) followed by Tukey's multiple comparison test using the Graph Prism 6.0 software. The values were considered significant when $p < 0.05$. Correlations were determined using Pearson's Coefficient (ρ) and classified as very strong ($\rho \geq 0.9$), strong ($0.7 \leq \rho \leq 0.89$), moderate ($0.5 \leq \rho \leq 0.69$), weak ($0.3 \leq \rho \leq 0.49$) and negligible ($\rho \leq 0.29$) [33].

3. Results

3.1. Growth and production of lactic acid by *Lactobacillus rhamnosus* in cupuaçu juice.

We firstly analyzed the growth of *L. rhamnosus* ATCC 9595 in Cupuaçu juice (120 mg/mL). This strain was able to grow in the juice without the addition of any supplement. Further, the viability of these strains was kept after 28 days of storage at 4 °C (Figure 1A). Based on these preliminary results we evaluated the effects of inoculum concentration (x_1) and the pulp concentration (x_2) in the growth and organic acids production by *L. rhamnosus* ATCC 9595.

In all assays, *L. rhamnosus* ATCC 9595 produced organic acid (as seen by reductions on pH media) displaying G/pH ratios ranging from 1.36 and 2.45 (Table 2). The best results were observed in the conditions used in the assays 4 and 6. These data were used to generate the surface response graph with a linearity coefficient (R^2) of 0.88661 (Figure 1B). Both studied variables significantly influenced the G/pH ratios ($p < 0.05$), however the inoculum concentration had the most important effect. This was also

evidenced by ρ values that indicated strong (ρ= 0.74) and weak (ρ= 0.47) correlations between G/pH ratios and inoculum and pulp concentrations, respectively.

Regarding the production of lactic acid, the yields ranged from 1.32 g/L to 5.72 g/L (Table 2). The surface response is exhibited a R^2 of 0.74475 (Figure 1C). In this case, the tested variables did not have significant impact on lactic acid levels ($p > 0.05$). However, a moderate correlation was observed by Pearson coefficient among inoculum concentration and lactic acid production (ρ= 0.65), while the correlation was negligible for pulp concentration (ρ= 0.47). The higher levels of lactic acid were found in assays 6 and 4. We selected the conditions of assay 4 to perform the animal assays.

3.2. The administration of *L. rhamnosus*-fermented juice reduced the severity of LPS-mediated endotoxemia

We used the median severity score (SS_{median}) to evaluate the progression of the LPS-mediated endotoxemia (Figure 2A). In the first 72 h, the LPS-inoculated animals without juice treatment showed higher severity scores (SS_{median} ranging from 9.0 to 6.0) than the health mice (those that received only PBS) (SS_{median}= 4.0). After 72h, all the groups exhibited SS_{median} equal to 4. The short-time intake of both unfermented and *L. rhamnosus*-fermented juice exhibited score significantly lower ($p<0.0001$) than untreated-endotoxemic group in this period. Importantly, the treatment with *L. rhamnosus*-fermented juice (SS_{median}= 4.0) also significantly improved the outcome of disease in relation to the group treated with unfermented cupuaçu juice (SS_{median}= 5.0) ($p<0.0001$). These positive effects of fermentation are clearly observed by the analysis of data from the calculation of Area under curve (AUC) (Figure 2B).

3.3. The administration of *L. rhamnosus*-fermented juice reduced the weight drop associated with endotoxemia induced by LPS

The induction of endotoxemia was also confirmed by measurement of body weight and temperature. As expected, the animals had a marked reduction on body weight (Figure 2C and 2D) and hypothermia (Figure 2E and 2F) after LPS injection. Regarding to body weight, the maximum reduction was observed after 48 h for all groups (ranging from 13.58% to 17.63%). The mice treated with *L. rhamnosus*-fermented juice exhibited the lowest values of body weight reduction in all evaluated periods, however significantly

differences were only detected after 48 h ($p<0.05$), 72 h and 96 h ($p<0.01$ for both) when compared with endotoxemic animals (Figure 2C). The data from AUC analysis confirmed these beneficial effects of fermented cupuaçu juice (Figure 2D). The treatment using unfermented cupuaçu juice did not have effect on body weight drop.

The three groups exposed to endotoxemia presented higher levels of hypothermia when compared with health animals (Figure 2E and 2F). However, significant differences were only observed among the control groups (LPS-untreated vs health animals). Although the mouse treated with *L. rhamnosus*-fermented juice displayed the lower levels of hypothermia, it was not possible to observe significantly differences with the other experimental groups.

3.4. The administration of *L. rhamnosus*-fermented juice amended the weight reduction of some organs associated with endotoxemia induced by LPS

The weight of some organs was also evaluated after 6 h of endotoxemia induced by LPS (Figure 3). The endotoxemia was associated with significant reductions in the weight of spleen (Figure 3A; $p<0.0001$), liver (Figure. 3B; $p<0.0001$), gut (Figure 3C; $p<0.05$) and kidneys (Figure 3D; $p<0.$ 0.0001). The treatment with *L. rhamnosus*-fermented juice significantly reduced the weight of spleen (Figure 3A; $p<0.0001$), liver (Figure 3B; $p<0.01$), gut (Figure 3C; $p<0.05$) and kidneys (Figure 3D; $p<0.0001$), when compared with endotoxemic group. In turn, mice treated with unfermented juice also exhibited reductions in spleen (Figure 3A; $p<0.0001$) and kidneys weights (Figure 3D; $p<0.0001$). Other organs (lung, brain, stomach) did not present significant differences after 6 h of endotoxemia induction. No treatment related alteration was observed for organs weights after 120 h (data not shown).

3.5. The administration of *L. rhamnosus*-fermented juice amended the migration of cells to peritoneal cavity in mice submitted to endotoxemia

Following, the peritonitis was evaluated by the migration of cells to peritoneal cavity (Figure 4). As expected, the endotoxemia induction significantly increased the number of leukocytes in relation to health animals (3.49-folds and 5.21-folds increased for 6 h and 120 h, respectively) (Figure 4A and 4B). The peritoneal fluids of endotoxemic

mice also exhibited higher counts of leukocytes after 6 h and 120 h of LPS injection than the other animals treated with *L. rhamnosus*-fermented or unfermented juices (Figure 4).

After 6 h, the number of total cells in PELF were reduced 3.39-folds by the short-term use of *L. rhamnosus*-fermented juice ($p<0.0001$) and 1.97-fold ($p<0.01$) in mice that received unfermented juice (Figure 4A). Similar, results were observed after 120 h of endotoxemia induction with reductions of 3.45-folds and 1.79-folds for groups administrated with fermented and unfermented juices, respectively (Figure 4B). At this period, the administration of *L. rhamnosus*-fermented juice significantly reduced the number of cells into peritoneal cavity in relation to the treatment with unfermented juice ($p<0.0001$).

Consequently, the administration of *L. rhamnosus*-fermented juice also significantly reduced the number of PMN cells in both evaluated periods when compared to untreated endotoxemic mice ($p<0.0001$; 7.57-folds and 4.68-folds after 6 h and 120 h, respectively) (Figure 4C and Figure 4D). Mice that received unfermented juice also displayed lower levels of PMN cells than LPS-untreated groups with reductions of 1.84-folds and 1.51-folds after 6 h ($p<0.01$) and 120 h, respectively. Significant differences between the number of PMN leukocytes among the groups treated with unfermented and fermented were only observed after 6 h of endotoxemia induction (Figure 4C; $p<0.05$).

The groups that received fermented and unfermented juices also had lower levels of MN leukocytes migration to peritoneal cavity after 6 h and 120 h of LPS inoculation than untreated endotoxemic animals (Figure 4E and Figure 4F). Once more, the higher rates of inhibition were seen for *L. rhamnosus*-fermented juice (reductions of 2.91-folds and 3.37-folds; $p<0.0001$) than unfermented juice (reductions of 2.03-folds and 1.81-folds; $p<0.0001$). Regarding the treatment, statistical differences were only observed after 120 h (Figure 4F; $p<0.01$).

3.6. The administration of *L. rhamnosus*-fermented juice reduced the increase of cells in blood associated with LPS-mediated endotoxemia.

We also evaluated the effects of fermented and unfermented cupuaçu juices in the population of cells in blood (Figure 5). The blood of endotoxemic animals showed higher number of circulating leukocytes than all other group in the analyzed periods ($p<0.0001$ for all groups) (Figure 5A and Figure 5B). The reductions were higher in the mice treated with *L. rhamnosus*-fermented juice (4-folds and 3.47-folds) than those treated with

unfermented juice (2.45-folds and 1.50-folds). However, significant different among the groups that received unfermented and fermented juice were observed only after 120 h (Figure 5B; $p<0.0001$).

The treatment with both fermented and unfermented juicy significantly reduced the levels of PMN leukocytes in the blood in relation to endotoxemic mice (Figure 5C and 5D; $p<0.0001$). Although the reductions in PMN cells were higher in mice treated with fermented juice (3.78-folds and 2.46-folds after 6 h and 120 h of endotoxemia induction, respectively) than those in those that received unfermented juice (3.19-folds after 6 h and 1.68-fold after 120 h), no significant differences were detected between the both types of treatment.

Finally, we analyzed the alterations in MN leukocytes in the blood of mice submitted to each treatment schedule. We observed that higher inhibitory effects for both type of treatment after 6 h of LPS-induction, with reductions of 4.01-folds and 2.42-folds for mice treated with fermented and unfermented juices (Figure 5E; $p<0.0001$). After 120 h (Figure 5F), the mice that received *L. rhamnosus*-fermented juice had the lower number of MN leukocytes in the blood in relation to both endotoxemic group (reduction of 4.20-folds; $p<0.0001$) and unfermented-treated mice ($p<0.01$). The treatment with unfermented mice resulted in a reduction of 1.43-fold when compared with endotoxemic mice ($p<0.05$).

4. Discussion

Despite many efforts, the deleterious consequences of endotoxemia remain as important causes of mortality [34,35]. This scenario denotes the urgent need for new alternatives to prevent and/or treat this condition [7,9]. Probiotics strains with immunomodulatory properties are indicated as attractive candidates for management of sepsis [13,14]. Herein, we show the beneficial effects of short-term intake of *L. rhamnosus*-fermented cupuaçu juice in a model of septic shock.

Several authors have indicated fruit juice as excellent vehicles for probiotic [36-38]. On the other hand, the fermentation can improve the activity of the phytochemicals present in the juice [39,40]. We demonstrate that cupuaçu juice was suitable for the growth of *L. rhamnosus* without any supplementation. Cupuaçu pulp has free sugars (glucose, fructose and sucrose) that supported the growth of *L. rhamnosus* and allowed the

production of lactic acid [36,41]. The production of organic acids can prevent the growth of possible pathogens [42,43].

Following, we evaluated whether the administration of cupuaçu fermented juice could improve the outcome of endotexemic shock. Animals models that mimics the metabolic alterations seen in endotoxemic individuals are usually used for preclinical evaluation of new therapeutic candidates [1], even though all these models have advantages and disadvantages in relation to translational applications [9,44]. An example of experimental model commonly used for mimicking the endotoxemic shock is the peritoneal administration of LPS, resulting in an exaggerated inflammatory response seen in endotoxin shock, followed by dysregulation of several organs [45,46].

Our results showed that both cupuaçu juice and cupuaçu juice fermented with *L. rhamnosus* were effective in reducing septic shock, with an anti-inflammatory profile. Some constituents such as ascorbic acid (vitamin C), tocopherols (vitamin E), flavonoids, anthocyanins and polyphenols provides antioxidant properties for this juice and may contribute to this action [26,27]. Natural phenolic compounds found in fruits and vegetables are known to exhibit anti-inflammatory effects [47,48]. These effects are possible to be observed by the reduction of proinflammatory cytokines such as TNF-α, IL-1 and IL-6, alteration of signal transduction in target cells, regulation of gene expression, in addition to modulation of antioxidant activity [49-51].

The most abundant compounds found in cupuaçu include polyphenol or flavonoid (proanthocyanidin) [25], whose potent antioxidant, immunomodulatory, anti-angiogenic and anti-proliferative activities have been demonstrated [52,53]. Butyric and hexanoic acids have also been found [54]. Butyric acid obtained through fermentation of probiotic bacteria demonstrated an anti-inflammatory effect in an inflammatory process due to epithelial damage [55].

L. rhamnosus strains have been showing ability to modulate the host immune system in different clinical situation [56,57]. The probiotic strains can also adhere to mucous membranes and provide protection against microbial infections that could be related to LPS-induced dysbiosis [58,59]. Additionally, *L. rhamnosus* strains are able to relieve hypersensitivity reactions and intestinal inflammation [60,61], being also indicated as an adjuvant in cases of neoplasms, eczema, diarrhea, lactose intolerance, intestinal inflammation and infections of the vaginal and urinary tracts [62-64].

In relation to *L. rhamnosus* ATCC 9595, this probiotic strain increased the survival time of *Galleria mellonella* larvae infected with *Candida albicans*. This action

was related to the inhibition of fungal virulence factors and modulation of the immune system as seen by the recruitment of hemocytes for hemolymph [65]. oreover, exopolysaccharides (EPS) produced by *L. rhamnosus* ATCC 9595 was able to exhibit immunosuppressive activity when tested on peritoneal macrophages of mice stimulated with LPS. The treatment with EPS induced the production of high levels of IL-10 and reduced the secretion of TNF-α [66].

Conclusion

In conclusion or cupuaçu juice, an ideal substrate for fermentation was *Lactobacillus rhamnosus* ATCC 9595, which showed a high viability in the optimized conditions. In addition, a probiotic bacterium was able to produce lactic acid in the required amount without the need to add supplements, having been used only using those that naturally contain fruit.

It was also possible to demonstrate that early therapy of fermented cupuaçu juice with *Lactobacillus rhamnosus* ATCC 9595 can reduce systemic inflammation in an experimental model of sepsis in mice.

These results may possibly help in the development of probiotic therapies in the treatment and prevention for patients with sepsis.

Table 1: Chemical composition of *T. grandiflorum* pulp

Sample	Humidity		Ash		Protein		Lipid		Carbohydrat*	
(*Theobroma*	%	DP	%	DP	%	DP	%	DP	%	DP
grandiflorum)	84,37	0,2	3,3	0,9	2,53	0,05	0,35	0,0	9,45	0,0

The results are means ± standard deviations (n = 3). * Calculated by difference = 100 - (protein + lipids + ash + moisture).

Table 2: Values of bacterial cycle tests (*L. rhamnosus*) and extracts (*T. grandiflorum*) and colony forming units (Log UFC.mL-1 / pH) for each experiment and lactic acid production (G / pH)

	Inocolum (OD_{600nm})	**Pulp (mg/mL)**	**Growth (Log CFU/mL)**	**pH**	**Latic acid (g/L)**	**G/pH ratio**	**G/[La] ratio**
1	1	163	6,13	4,5	1,32	1,36	4,65
2	1	297	7,32	4,4	3,96	1,66	1,85
3	2,1	163	8,41	4,9	3,25	1,72	2,59
4	2,1	297	9,07	3,7	4,98	2,45	1,82
5	0,77	230	7,29	4,7	0,65	1,55	11,25
6	2,33	230	8,46	3,9	5,72	2,17	1,48
7	1,55	135,25	7,44	4	1,44	1,86	5,17
8	1,55	324,75	9,43	4,6	1,13	2,05	8,34
9	1,55	230	8,67	4,2	1,25	2,06	6,92
10	1,55	230	9,19	4,5	1,14	2,04	8,05

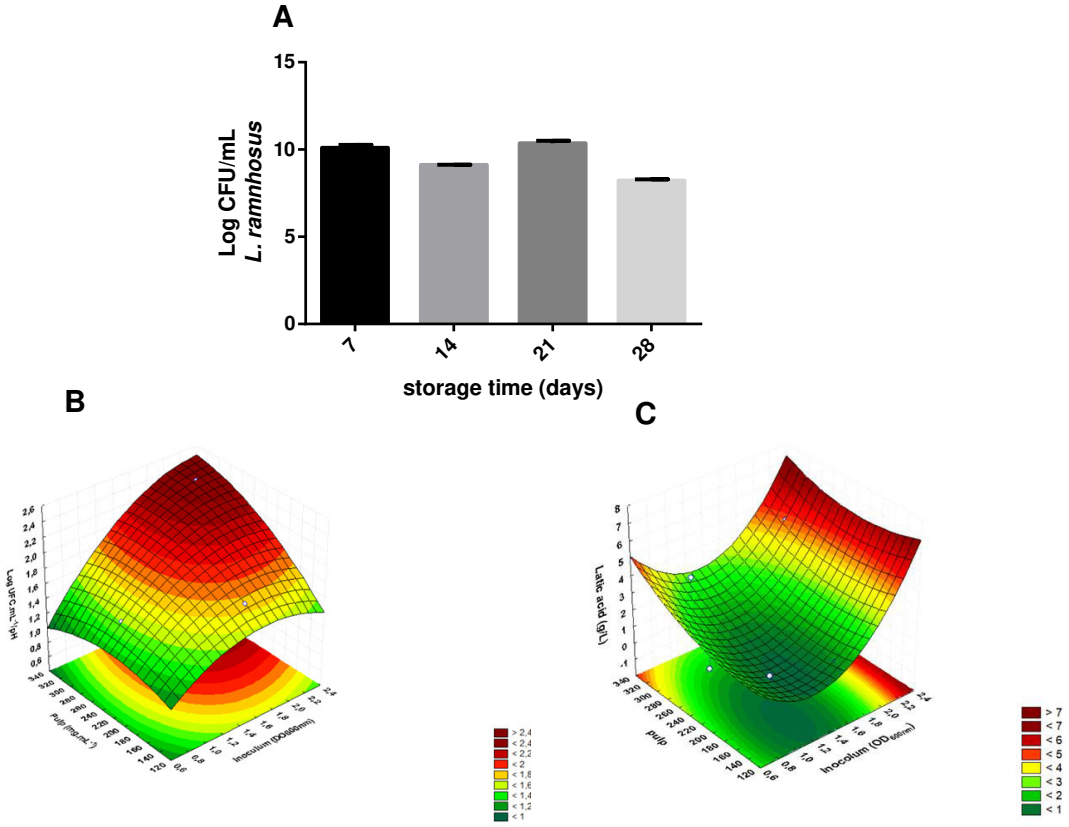

Figura 1: Growth and production of lactic acid by *Lactobacillus rhamnosus* in cupuaçu juice. (A) Survival of *L. rhamnosus* ATCC 9595 in the fermentations of cupuaçu juice by L. rhamnosus ATCC 9595 during the storage period. (B) Response surface obtained for RVpH as a function of pulp and inoculum concentrations in cupuaçu juice fermentations by *L. rhamnosus* ATCC 9595. (C) Response surface obtained for lactic acid concentration as a function of pulp and inoculum concentrations in cupuaçu juice fermentations by *L. rhamnosus* ATCC 9595.

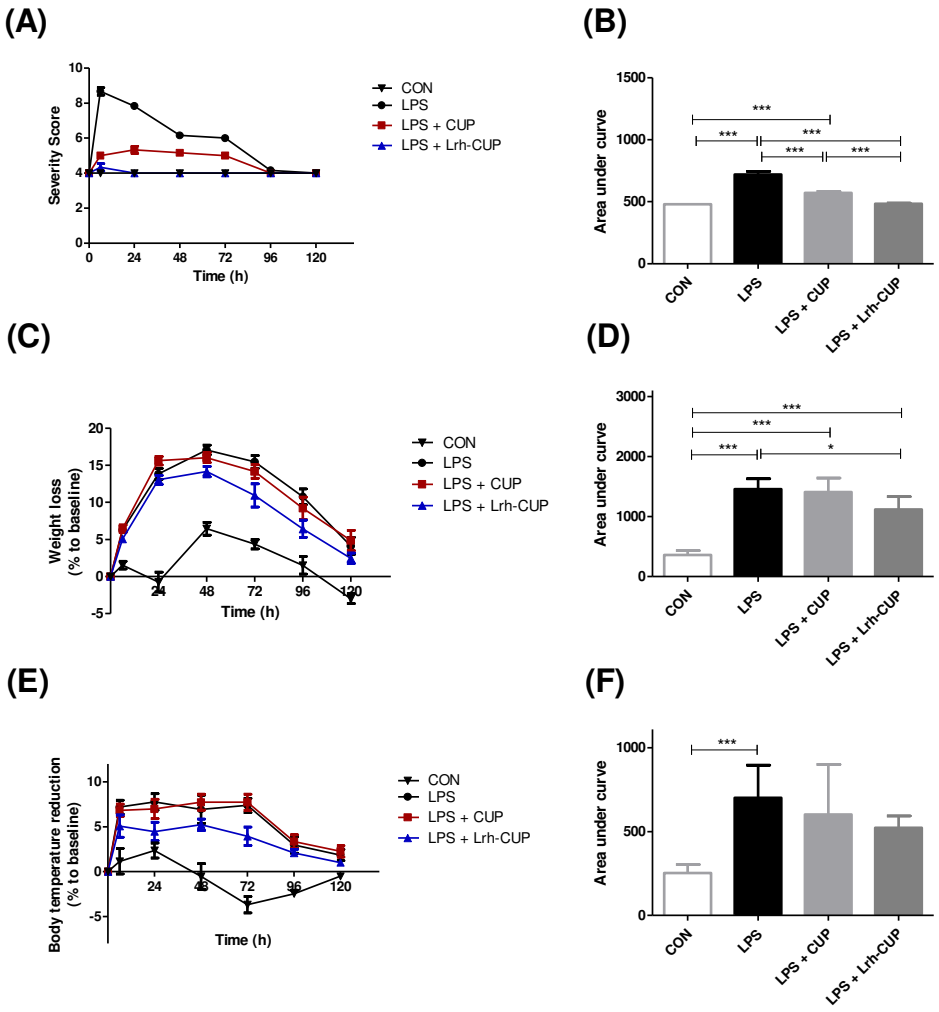

Figure 2: Effects of fermented and unfermented *Theobroma grandiflorum* juice on some pathologic parameter associated with LPS-mediated endotoxemia. (A) Kinects of variation of severity score; (B) Area under curve from data of severity score analysis; (C) Kinects of variation of weight loss; (D) Area under curve from data of Weight loss analysis; (E) Kinects of variation of body temperature reduction; (F) Area under curve from data of body temperature reduction analysis. *Significant differences with $p<0.05$; **Significant differences with $p<0.01$; ***Significant differences with $p<0.0001$. LPS: mice submitted to LPS-mediated endotoxemia; LPS + CUP: mice treated with unfermented *T. grandiflorum* juice and submitted to LPS-mediated endotoxemia; LPS + Lrh-CUP: mice treated with *L. rhamnosus*-fermented *T. grandiflorum* juice and submitted to LPS-mediated endotoxemia.

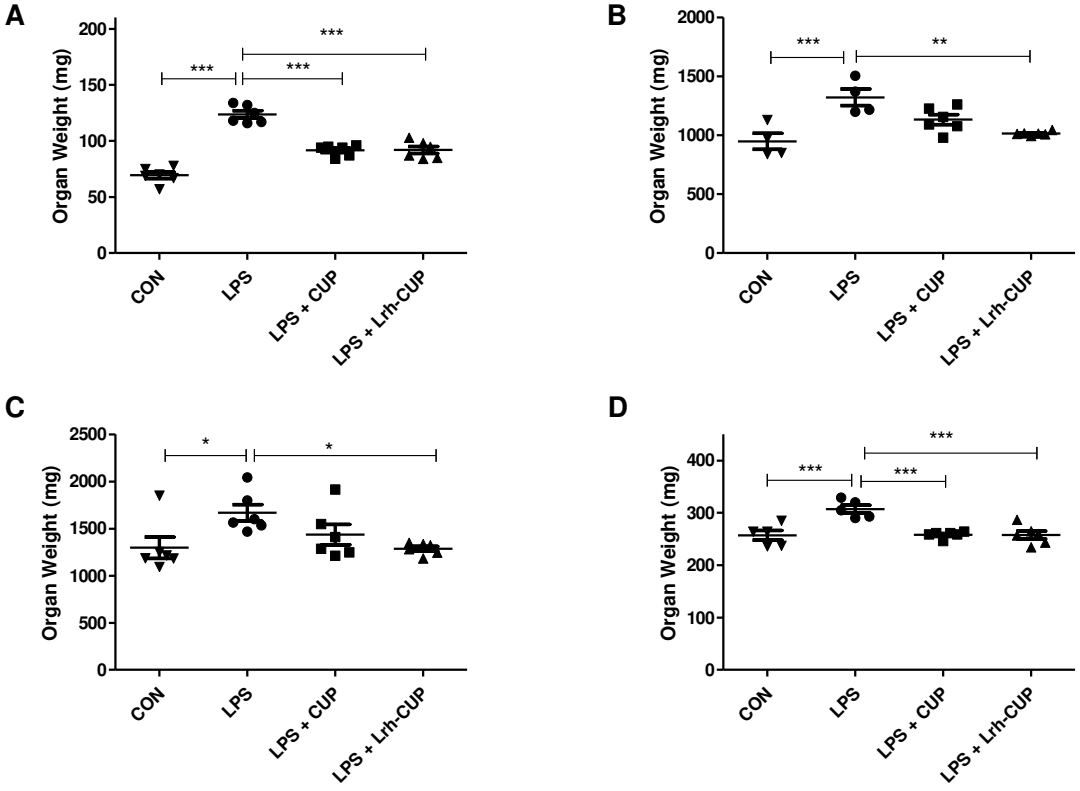

Figure 3: Effects of fermented and unfermented *Theobroma grandiflorum* juice on the weight of some organs of mice submitted to LPS-mediated endotoxemia. (A) Spleen; (B) Liver; (C) Gut; (D) Kidneys. *Significant differences with $p<0.05$; **Significant differences with $p<0.01$; ***Significant differences with $p<0.0001$. LPS: mice submitted to LPS-mediated endotoxemia; LPS + CUP: mice treated with unfermented *T. grandiflorum* juice and submitted to LPS-mediated endotoxemia; LPS + Lrh-CUP: mice treated with *L. rhamnosus*-fermented *T. grandiflorum* juice and submitted to LPS-mediated endotoxemia.

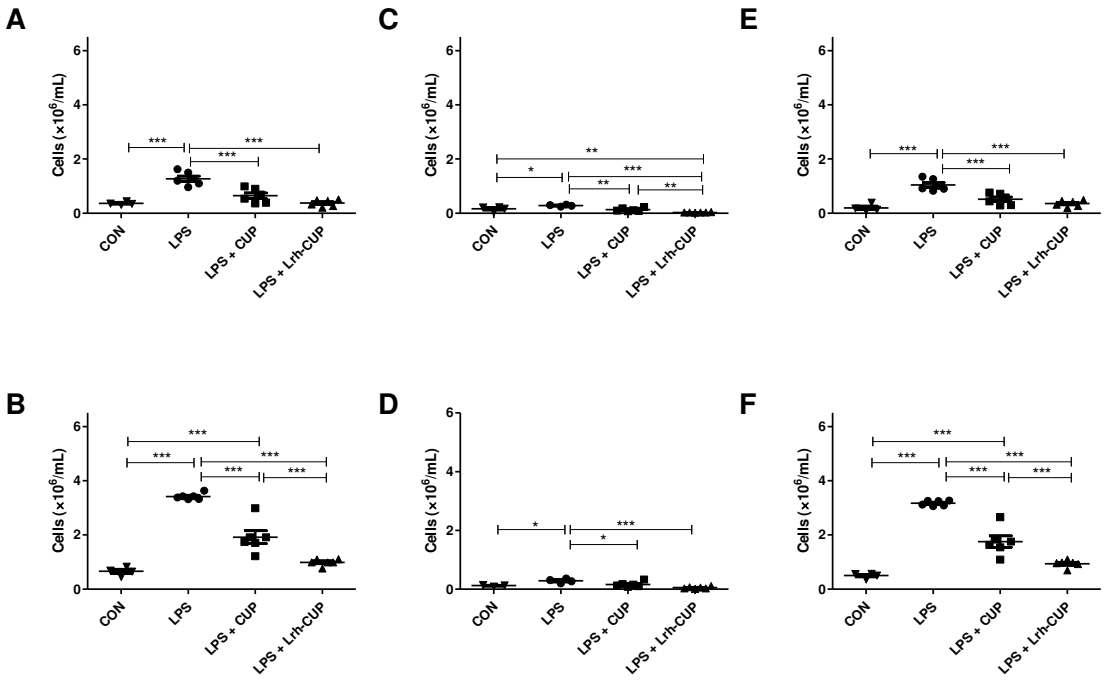

Figure 4: Effects of fermented and unfermented *Theobroma grandiflorum* juice in the migration of cells to peritoneal cavity in mice submitted to LPS-mediated endotoxemia. (A) Total leukocytes in the peritoneal cavity after 6 h of LPS-mediated endotoxemia; (B) Polymorphonuclear leukocytes in the peritoneal cavity after 6 h of LPS-mediated endotoxemia; (C) Mononuclear cells in the peritoneal cavity after 6 h of LPS-mediated endotoxemia; (D) Total leukocytes in the peritoneal cavity after 120 h of LPS-mediated endotoxemia; (E) Polymorphonuclear leukocytes in the peritoneal cavity after 120 h of LPS-mediated endotoxemia; (F) Mononuclear cells in the peritoneal cavity after 120 h of LPS-mediated endotoxemia. *Significant differences with $p<0.05$; **Significant differences with $p<0.01$; ***Significant differences with $p<0.0001$. LPS: mice submitted to LPS-mediated endotoxemia; LPS + CUP: mice treated with unfermented *T. grandiflorum* juice and submitted to LPS-mediated endotoxemia; LPS + Lrh-CUP: mice treated with *L. rhamnosus*-fermented *T. grandiflorum* juice and submitted to LPS-mediated endotoxemia.

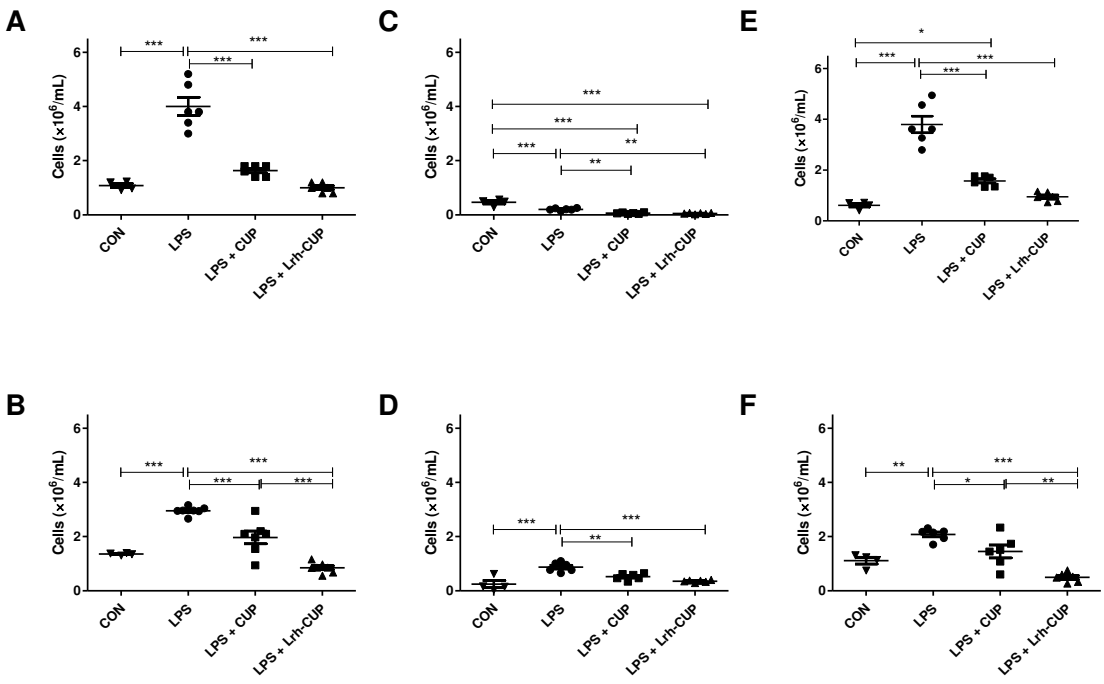

Figure 5: Effects of fermented and unfermented *Theobroma grandiflorum* juice in leukocytes population in the blood of mice submitted to LPS-mediated endotoxemia. (A) Total leukocytes in the peritoneal cavity after 6 h of LPS-mediated endotoxemia; (B) Polymorphonuclear leukocytes in the peritoneal cavity after 6 h of LPS-mediated endotoxemia; (C) Mononuclear cells in the peritoneal cavity after 6 h of LPS-mediated endotoxemia; (D) Total leukocytes in the peritoneal cavity after 120 h of LPS-mediated endotoxemia; (E) Polymorphonuclear leukocytes in the peritoneal cavity after 120 h of LPS-mediated endotoxemia; (F) Mononuclear cells in the peritoneal cavity after 120 h of LPS-mediated endotoxemia. *Significant differences with $p<0.05$; **Significant differences with $p<0.01$; ***Significant differences with $p<0.0001$. LPS: mice submitted to LPS-mediated endotoxemia; LPS + CUP: mice treated with unfermented *T. grandiflorum* juice and submitted to LPS-mediated endotoxemia; LPS + Lrh-CUP: mice treated with *L. rhamnosus*-fermented *T. grandiflorum* juice and submitted to LPS-mediated endotoxemia.

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CAPÍTULO 2

Evaluation of growth and viability of probiotic bacteria (*Limosilactobacillus fermentum* ATCC 23271 and *Lacticaseibacillus rhamnosus* ATCC 9595) in bacuri juice (*Platonia insignis*)

1. Introduction

Functional foods containing probiotics have been demonstrating effectiveness to prevent or treat health problems, including digestive disorders, such as irritable bowel syndrome and necrotizing enterocolitis [1-3]. In particular, those formulations containing *Lactobacillus* sp. are recognized for their ability to modulate the human microbiota and induce anti-inflammatory and antioxidant effects [4-6].

Probiotics are commonly found in dairy foods, such as milks, yogurts and cheeses [4,7,8]; however, fruit juices may be excellent vehicles for development of probiotic products due their nutritional properties and presence of bioactive compounds (vitamins, phenolic acids, flavonoids and other antioxidant compounds) [9-11]. The use of fruit juices containing probiotics also gives opportunities individuals with specific conditions (lactose intolerance, allergy to milk components and vegetarians) to benefit from the consumption of these bacteria [12,13].

Brazil is one of the countries that has the largest repertoire of fruits in the world, constituting a comprehensive amount of tropical and exotic fruits with unique aromas and flavors [14]. An example is Bacuri, the fruit of the species *Platonia insignis* Mart. (Clusiaceae), popularly known in Brazil as bacurizeiro. Bacuri is a round fruit, with thick skin, and a citrus-yellow color. It contains a very tasty viscous pulp, used in sweets, liqueurs and ice cream [15,16].

The antioxidant potential and α-glucosidase inhibitory of fruit pulp has been demonstrated [17]. In fact, the abundance of bioactive compounds (such as citric acid, p-cumaric acid and terpenes) present in bacuri has the ability to potentiate the beneficial effects of probiotic bacteria [17,18]. Additionally, the presence of sugars (glucose, fructose and sucrose), vitamins (C, E) and metals (Na, K, Ca, Mg, P, Fe, Zn and Cu) [19] make its juice an interesting vehicle for the development of fermented products.

In this context, this work evaluated the growth of two strains of *Lactobacillus* with probiotic characteristics such us *Limosilactobacillus fermentum* ATCC 23271 previously

called and *Lacticaseibacillus rhamnosus* ATCC 9595 previously called *Lactobacillus rhamnosus* grown in *P. insignis* juice. The effects of juice cultivation on the production of lactic acid by *L. rhamnosus* ATCC 9595, as well as its chemical resistance and anti-infective properties were also evaluated. Finally, we analyzed the protective effects of the fermented juice in a model of infection induced by enteroaggregative *Escherichia coli* EAEC in *Tenebrio molitor* larvae.

2. Material and methods

2.1. Origin and maintenance of probiotic strains

The strains of *L. fermentum* ATCC 23271 and *L. rhamnosus* ATCC 9595 used in this study were obtained from the Microbial Colletion of the Ceuma University. The strains are kept refrigerated at -80 ° C. For the experiments, aliquots were activated in MRS broth (De Man, Rogosa and Sharpe). The analyzes to determine the proximate composition of *P. insignis* were carried out in triplicate [20]. The Nifext fraction (carbohydrates) was obtained by calculating the difference from the other fractions analyzed. The composition of fatty acids was determined by the Soxhlet method, as well as the determination of moisture and ash were performed as described in the physical-chemical methods for food analysis [21] (Table 1).

2.2. Obtaining the fruits of *P. insignis*

The fruits of *P. insignis* were collected in the Cerrado Maranhense region, in the south of the state, which comprises the mesoregions of Balsas, Estreito, Carolina, Porto Franco and Riachão. The fruit pulp was manually removed and stored at -20ºC until its preparation. Identification number 11.540.

2.3 Initial fermentation and viability tests under refrigeration

In the initial fermentation tests, an aliquot of pulp (30 g) were dissolved in 250 mL of distilled water (concentration of 120 mg/mL). The pH of the juice was adjusted to 6.0 before sterilization by autoclaving. In parallel, a pre-inoculum for each bacterium (*L. fermentum* ATCC 23271 or *L. rhamnosus* ATCC 9595) was prepared in MRS broth. Probiotics were grown at 37 ° C under agitation (120 rpm). After 24 h, 1 mL aliquots of

each bacterial suspension [optical density at 600 nm (OD_{600nm}) = 1.0] were inoculated in bacuri juice or in an MRS broth. The flasks were incubated with shaking at 120 rpm for 48 hours.

The quantification of bacterial growth was performed by plating on MRS agar. Serial dilutions were made in PBS solution after each determined period (0, 7, 14, 21 and 28 days of refrigeration). Then, the Petri dishes were incubated for 48 hours at 37 °C. The number of bacteria was determined by counting colony-forming units (CFU) and expressed in CFU/mL.

2.4 Optimization of cultivation conditions

The optimization of the cultivation conditions was performed through a Central Rotational Composite Design (CCRD). The two variables chosen were the inoculum concentration (x^1) and the pulp concentration (x^2), with a total of 10 experiments (Table 2). After each test, the pH values and the microbial population were analyzed to determine the relationship between bacterial growth and pH (G/pH), as well as better production of lactic acid [22].

2.4.1 Quantification of lactic acid contente

The quantification of lactic acid in the fermentative liquid was performed as described in [22], using a Shimadzu high-performance liquid chromatograph, equipped with a quaternary pump r coupled to a degassing system (DGU-20A5r). The system contains an oven to control the column temperature (set at 28 ° C) and an automatic injector (20 µL injection) with a diode array detector (SPD-M20A; range 190-800 nm). An ion exchange column (300 mm x 7.8 mm x 9 µm; Aminex® HPX-87H, Bio-Rad, USA) was used. The elution was carried out isocratically with a mobile phase composed of 5 mM H_2SO_4 and with a flow of 0.6 mL/min. The software used was LC-Solutions manufactured by Shimadzu Corporation (Kyoto, Japan).

For each test, the concentration of lactic acid in an unfermented juice containing the same concentration of pulp (unfermented controls) was also detected. The production of lactic acid (g/L) was determined by the difference between the concentration of lactic acid in each fermented liquid and its respective unfermented control.

2.5 Effect of juice in bacterial tolerance towards lisozime and pH

Tolerance tests were performed as described by Singhal (2010) with modifications. In the first trial lysozyme (Sigma-Aldrich, USA) was added to the MRS broth or to the juice (230 mg / mL of pulp) to reach a concentration of 300 µg / mL. These solutions were transferred (100 µL) to 96-well microplates and, subsequently, 10 µL of the fermented juice was added under the conditions of run 6. Similarly, in the acidity tolerance tests, the pH of the juice or medium was adjusted to reach pH values of 3 or 4. These solutions were transferred (100 µL) to 96-well microplates and, subsequently, 10 µL of the fermented juice was added under run 6 conditions. After each assay, the plates were incubated for 3 h and then plated on MRS agar. The determination of the bacterial population was determined after 48 h of incubation.

2.6 Enteroaggregative *Eschericia coli* infection test in larvae of *Tenebrio molitor*

The infection model using *T. molitor* larvae was used to determine the effectiveness of fermented or non-fermented juices in inhibiting infection provoked by Enteroaggregative *Escherichia coli* EAEC 042 (EAEC 042) [23]. The juices were inoculation of the samples before (preventive) and after infection (treatment). In all experiments, the larvae (~ 100 mg) were anesthetized and disinfected (in ice and 70% alcohol, respectively) and randomly allocated to groups (n = 10 / group). The infection was established by inoculation of 10 µL of standardized EAEC 042 suspension (OD_{600nm} = 0.1) at the membrane between the second and third abdominal annular segment (in the tail-head direction).

In the groups submitted to treatment, the 10 µL aliquots of the fermented and non-fermented juices (filtered through a 0.22 µm membrane) were inoculated 2 h after infection. On the other hand, to assess the preventive effect, aliquots of juices were administered 2 h before infection. Animals inoculated with phosphate-saline buffer (PBS, pH 7.4) were used for positive viability control. Larval survival was analyzed daily.

2.7 Statistical analysis

The experiments were carried out in triplicate and in three independent tests. All results were expressed as mean values and were analyzed considering the value of $p < 0.05$ as statistically significant. The data were analyzed using the GraphPad Prism® (version 7.0) or Statistica software. Correlations were determined using Pearson's Coefficient (ρ) and classified as very strong ($\rho \geq 0.9$), strong ($0.7 \leq \rho \leq 0.89$), moderate ($0.5 \leq \rho \leq 0.69$) , weak ($0.3 \leq \rho \leq 0.49$) and negligible ($\rho \leq 0.29$) [24]. Survival tests were analyzed using the Kaplan – Meier method and the Log-rank test.

3 Results and discussion

3.1 Growth and viability after storage of *Lactobacillus* strains in *Platonia insignis* juice

The first step of the work was to evaluate whether the *Lactobacillus* strains had the capacity to grow in the juice of *P. insignis* (Figure 1). In both cases, a significantly higher growth was observed in bacuri juice (without the addition of nutritional supplements) than in the MRS medium ($p < 0.05$). In the case of *L. fermentum* ATCC 23271, the average difference between growth in *P. insignis* juice and MRS medium was 2.84 Log CFU/mL (Figure 1A; $p < 0.05$); while the difference was 7.39 Log (Figure 1B; $p < 0.001$) for *L. rhamnosus* ATCC 9595.

Following, it was analyzed the viability of each strains during the storage in the juice at 4 °C up to 28 days (Figure 1C). During this period, the lactobacilli remained viable with similar variation after 28 days (around 30% for both). However, the colony countings were significantly higher for *L. rhamnosus* ATCC 9595 (7.40 ± 0.04 Log CFU/mL) than *L. fermentum* ATCC 23271 (6.29 ± 0.12 Log CFU/mL) ($p < 0.0001$). It is important to highlight that the viability levels at the end of the storage time were above the minimum limit (10^6 CFU/mL; 15 to 30 days) for probiotic-containing products. Taken together, these data evidenced that the juice of *P. insignis* would be a good probiotic food matrix.

In general, these results are similar to those found for other juices [25,26]. The explanation for growth and high viability of these probiotic during storage strains in bacuri juice may be the chemical compostion of this pulp with avaibility of amino acids (leucine, glutamine, arginine, alanine, valine, isoleucine), vitamins, minerals and carbohydrates (glucose, fructose and sucrose) that correspond up to 50% of dry matter)

[27]. Further, the phenolic compounds from bacuri may be fermented by these bacteria and promote their growth [28].

3.2 Effect of seletected variable on bacterial growth and acid production

L. rhamnosus ATCC 9595 was selected for the assays of optimization of the cultivation conditions through an experimental design. In all conditions evaluated, *L. rhamnosus* ATCC 9595 was able to grow (average ranging from 7.52 ± 0.09 Log CFU/mL to 10.22 ± 0.09 Log CFU/mL) and decrease the pH values (marker of organic acid production) from 6.0 to values ranging from 3.9 to 5.0 was observed. The G/pH ratio values ranging from 1.53to 2.13 (table 2).

The surface response plot showed in Figure 2A illustrate the influence of the two selected independent variables (inoculum and *P. insignis* pulp concentrations) in the values for G/pH ratio. A linearity coefficient (R^2) of 0.87 was found and the curve can be described with the equation: $z = 0.14767228670735 + 1.3205979104405x - 0.5332911196835x^2 + 0.0081505369003327y - 0.000026303871055073y^2 + 0.0027846725180107xy$. The most favorable conditions were those used in experiment 6 (G/pH = 2.13) and at the central points (assays 9 and 10; G/pH = 2.11 and 2.08). In these tests the same concentration of pulp (230.00 mg/mL) with different inoculum densities (2.33 and 1.55, respectively) (Table 2).

It was observed that the inoculum concentration is the main factor to obtain more favorable values of G/pH ratio ($p < 0.05$). This positive influence of the inoculum is evidenced by the analysis of Pearson's coefficient (ρ), which indicates a strong correlation between the values of the inoculum and the G/pH ratios ($\rho = 0.70$); while the pulp concentration had a negligible correlation with these indexes ($\rho = 0.10$).

The positive effects of inoculum concentration on G/pH ratio was also observed for the optimization of *L. rhamnosus* ATCC 7469 in *Passiflora cincinnata* juice (Caatinga passion fruit). The study also reported similar values of bacterial growth (8-10 Log CFU/mL) that those observed in bacuri juice [22]. Regarding to pH variation, *Lactobacillus* strains require slightly acidic pH for maximum growth [29]. The pH of bacuri is around 3.12 to 3.48 [30], thus, we neutralized it (pH ~ 6.0) by 1M NaOH as a food additive authorized by Brazilian legislation (ANVISA 2007; COSTA et al., 2013). The reduction of pH during the fermenation is an indicative of organic acid production and growth [29,31,32].

3.3 Effect of selected conditions on lactic acid production

The influence of the selected independent variables (inoculum and pulp concentrations) on the production of lactic acid by *L. rhamnosus* ATCC 9595 was also evaluated. The average initial concentration of lactic acid in *P. insignis* pulp was 0.18 ± 0.02 mg/g. The variation in the production of lactic acid was 1.32 g/L to 4.14 g/L (table 2), with the best yields being obtained in tests 6 and 8 (inoculum at 1.55 and pulp at 324.75 mg/mL) with concentrations of 4.14 g/L and 3.98 g/L of lactic acid, respectively. The response surface generated with these data is represented in figure 2B.

The independent variables on lactic acid production had no influence on lactic acid production ($p > 0.05$). Pearson's Coefficient analysis indicated that the inoculum concentration has a moderate correlation ($\rho = 0.50$) with acid production, while the concentration of the pulp has a weak correlation ($\rho = 0.43$). A moderate correlation was also observed between the production of lactic acid and bacterial growth ($\rho = 0.66$).

We also evaluated the relation between bacterial growth and latic acid production (G/[La] ratio) (Fig. 2C). Again, the most favaroable results were observed for the conditions of assays 6 and 8 (2.47 and 2.20) (table 2). Although the variables did not significantly impact the G/[La] ratio, we could find moderate correlation among G/pH and G/[La] ratios ($\rho = 0.50$). Based in our results we decide to select the conditions used in assay 6 for perform the further evaluations.

The production of organic acids by *Lactobacillus* strains depends on the composition of the media [33]. In our study, the avaliable of nitrogen and carbon sources in bacuri juice were sufficient for synthesis of lactic acid by *L. rhamnosus* ATCC 9595 comparable to other fermented juice. For instance, *L. rhamnosus* HN001 produced higher level of lactic acid (4.4 g/L) when cultivated in star fruit (*Averrhoa carambola*) juice [34].

3.4 Effects of cultivation of *L. rhamnosus* ATCC 9595 on juice on resistance to simulated conditions of the Gastrointestinal Tract

The modulation of the gastrointestinal tract by probiotic bacteria depends on their ability to resist the adversities of this ecosystem (acidity and the presence of enzymes). Therefore, this characteristic should be evaluated each probiotic candidate and their formulation. *L. rhamnosus* ATCC 9595 is reported to completely tolerate pH values ≥ 2.5 in a simulated gastrointestinal fluid containing pepsin and NaCl [35].

Two assays were performed to assess whether the growth in *P. insignis* juice would alter the ability of *L. rhamnosus* ATCC 9595 to tolerate conditions found in the gastrointestinal tract (Figure 3). In both tests (pH tolerance and lysozyme) the bacteria showed similar results for growth in MRS medium and *P. insignis* juice ($p > 0.05$). Bacterial quantification values ranged from 7.03 ± 0,05 Log CFU/mL to 5.15 ± 0,05 Log CFU/mL for pH 4, and from 6.27 ± 0,04 *P. insignis* Log CFU/mL to 5.01 ± 0,05 Log CFU/mL to pH 3, when grown in MRS medium and *P. insignis* juice, respectively (Figure 3A). In the tolerance test, there was a bacterial growth of 7.05 ± 0,07 Log CFU/mL in the MRS medium, whereas in bacuri juice the bacterial growth was 5.16 ± 0,13 Log CFU/mL (Figure 3B).Our results show similar viability profile of *L. rhamnosus* ATCC 9595 when cultivated in MRS medium of bacuri juicy modified by acidification or addition of lysozyme. These data should be further confirmed in *in vivo* analysis.

3.5 Effects of fermented and non-fermented juices on enteroaggregative *Escherichia coli* infection in larvae of *Tenebrio molitor*

Finally, the ability of fermented and non-fermented juice to alter the course of infection by EAEC 042 in *T. molitor* larvae was evaluated (Figure 4). This organism has been used as alternative model for study microbial pathogenesis and prospection of anti-infective agents. In our assay, infected larvae without treatment had a median survival of 2 days, while uninfected larvae remained alive throughout the experimental period (8 days). The survival curves of these two groups were significantly different ($p < 0.001$).

The pre-treatment with supernatants of both fermented and non-fermented juices showed anti-infective effects with a significant increase in larval survival, in relation to untreated infected larvae (Figure 4A; $p < 0.05$). The survival percentages for animals pre-treated with juices at the end of the period were greater than 60%. The survival curves for infected and pre-treated larvae with the two types of juices did not show significant differences ($p > 0.05$). Further, we evaluated the effects of the inoculation of juices two hours after infection by EAEC 042 (Figure 4B). Larvae treated with fermented juice showed a higher percentage of survival at the end of treatment (40%; median survival of 2.5 days) than the other infected groups (20% and median survival of 2 days).

Compounds with antimicrobial and anti-inflammatory potentials have been reported in Bacuri pulp which may explain these anti-infective effects observed for unfermented juice. Similarly, some strains of *L. rhamnosus* exhibithed inhibitory activity

against *E. coli* infection [36,37]. Specifically, the administration of *L. rhamnosus* ATCC 9595 reduced the countings for total Enterobacteriaceae in mice feaces. This strain is also pointed as promisse antagonist agent towards *Candida albicans*, inhibiting the expression of virulence determinats (biofilm formation and filamentation) and protecting *Galleria mellonella* larvae [38,39].

4 Conclusion

Bacuri juice demonstrates an alternative substrate for probiotic cultures, as it maintains the viability, growth and production of vegetables. The fermented juice is kept stable and viable after 28 days of refrigerated storage without the addition of a supplement. In addition, a probiotic bacterium was able to produce lactic acid in the required amount without the need to add supplements, having been used only using those that naturally contain fruit.

In *in vivo* model or preventive treatment with supernatants from fermented and unfermented juices, it has anti-infectious effects with a significant increase in larval survival, in relation to untreated infected larvae.

Lacticaseibacillus rhamnosus ATCC 9595, grown without juice, increases growth in simulated conditions of the gastrointestinal tract, however, it suggests using an encapsulation or cultivating probiotic and cultivating probiotic and prebiotic together, without increasing bacterial viability in these adversities. The results obtained in this research allowed the basis for the development of a non-dairy probiotic product from Bacuri juice.

Table 1: Chemical composition of *P.insignis* pulp

Sample	Humidity		Ash		Protein		Lipid		Carbohydrat*	
(*Platonia insignis)*	%	DP	%	DP	%	DP	%	DP	%	DP
	81,67	0,3	0,52	0,02	3,9	0,1	0,10	0,0	13,81	0,0

The results are means ± standard deviations (n = 3). * Calculated by difference = 100 - (protein + lipids + ash + moisture).

Table 2: Values of bacterial cycle tests (*L. rhamnosus*) and extracts (*P. insignis*) and colony forming units (Log UFC.mL-1 / pH) for each experiment and lactic acid production (G / pH)

	OD_{600nm}	Pulp mg/mL	Growth	Final pH	Ácido Láctico (g/L)	G/pH ratio	G/[La] ratio
1	1.00	163.00	7.95 ± 0.10	4.80	1.32	1.66	6.02
2	1.00	297.00	7.52 ± 0.09	4.90	1.88	1.53	4.00
3	2.10	163.00	8.48 ± 0.04	4.90	1.94	1.73	4.37
4	2.10	297.00	9.49 ± 0.04	4.70	2.06	2.02	4.61
5	0.77	230.00	7.85 ± 0.02	5.00	1.97	1.57	3.98
6	2.33	230.00	10.22 ± 0.09	4.8	4.14	2.13	2.47
7	1.55	135.25	7.53 ± 0.06	3.90	2.12	1.93	3.55
8	1.55	324.75	8.78 ± 0.05	4.50	3.98	1.95	2.20
9	1.55	230.00	8.86 ± 0.04	4.20	2.48	2.11	3.57
10	1.55	230.00	8.72 ± 0.05	4.20	2.33	2.08	3.74

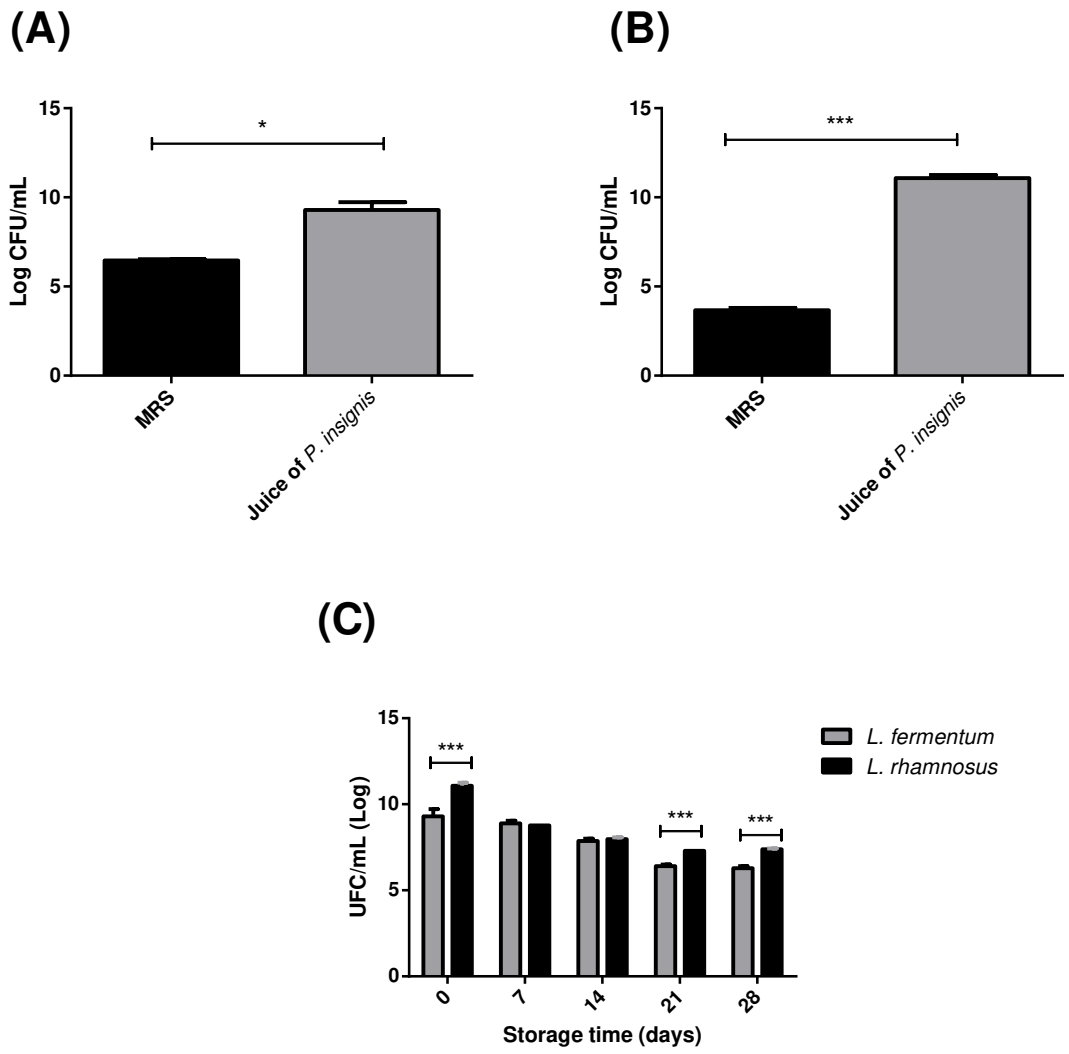

Figure 1: Evaluation of the growth of strains of *Lactobacillus* in the juice of *Platonia insignis* (bacuri). (A) Comparison of Limosilactobacillus fermentum growth in MRS Agar medium and in P. insignis juice after 48 h of incubation. (B) Comparison of *Lacticaseibacillus rhamnosus* growth in MRS Agar medium and in P. insignis juice after 48 h of incubation. * Statistical differences with $p < 0.05$; *** Statistical differences with $p < 0.001$. (A) Comparison of the effect of storage on P. insignis juice on the population of *L. fermentum* ATCC 23271; (B) Variation in the population of L. rhamnosus ATCC 9595 in *Platonia insignis* juice.

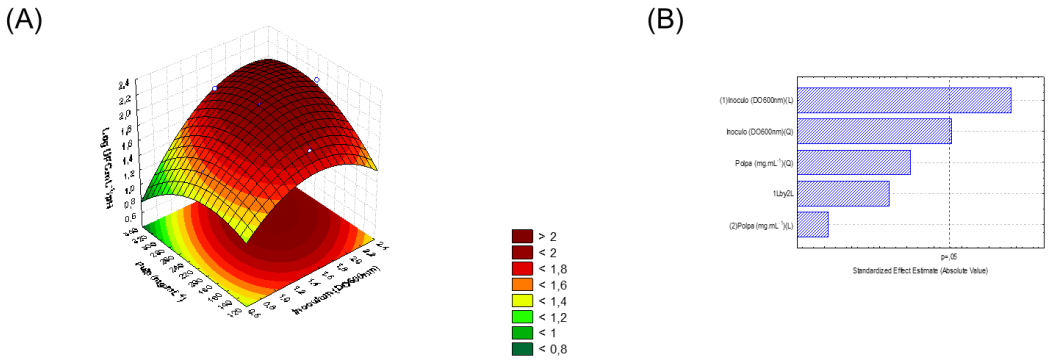

Figure 2: Effect of the concentration of the inoculum and the pulp of *Platonia insignis* on the growth and production of organic acids by L. rhamnosus ATCC 9595. (A) Response surface obtained for the Bacterial growth / pH ratio as a function of the studied variables. (B) Representation of the Pareto Chart.

(A) (B)

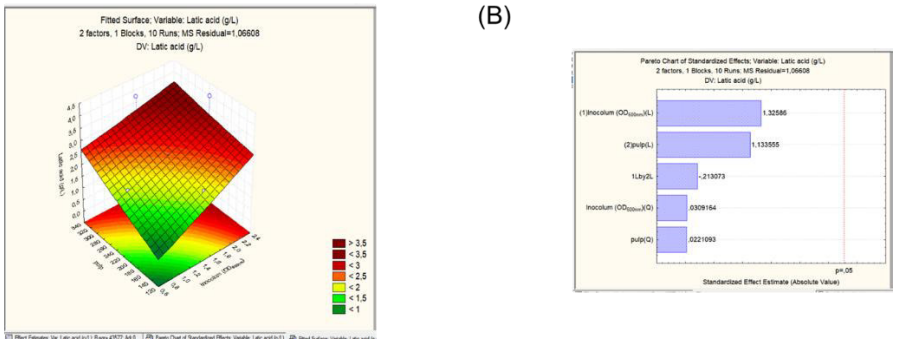

Figure 3: Effect of the concentration of the inoculum and the *Platonia insignis* pulp on the production of lactic acid by *L. rhamnosus* ATCC 9595. (A) Response surface obtained for the production of lactic acid as a function of the variables studied. (B) Representation of the Pareto Chart.

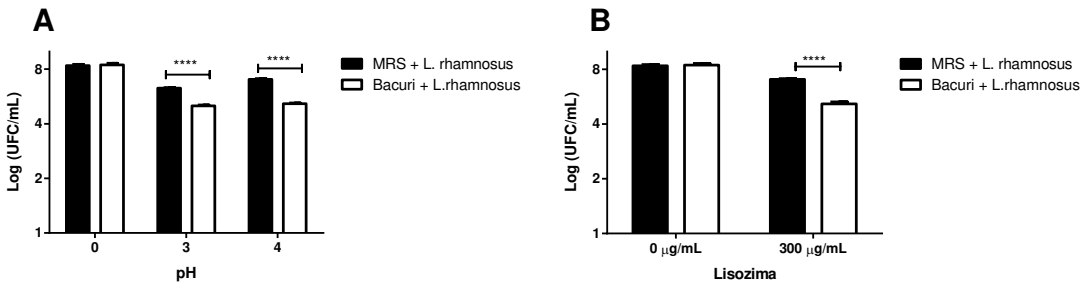

Figure 5: Test of tolerance to simulated conditions of the Gastrointestinal Tract.
(A) pH tolerance test. (B) Lysozyme tolerance test.
Man Rogosa and Sharpe – MRS, *Lacticaseibacillus rhamnosus – L. rhamnosus*

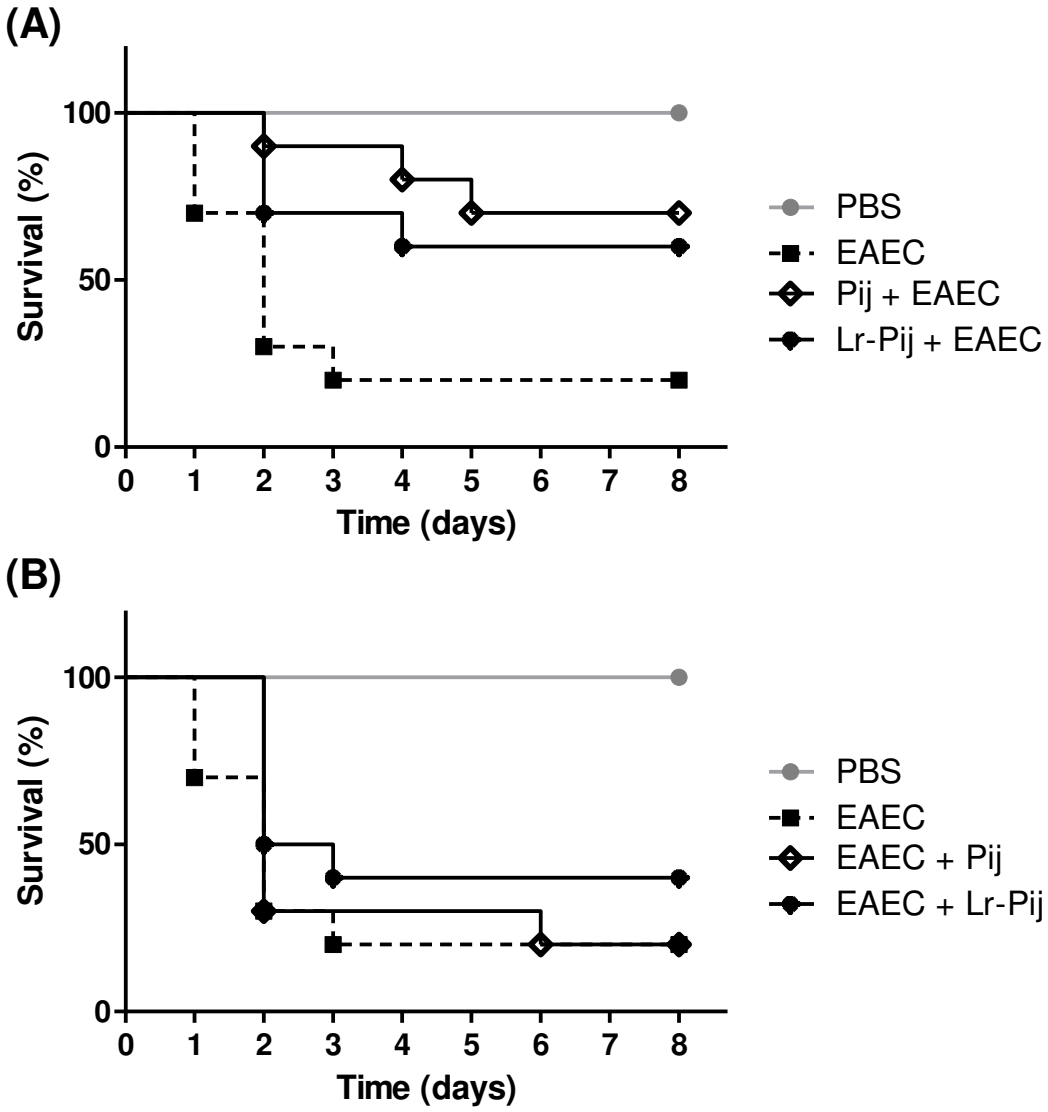

Figure 6: Effects of fermented and non-fermented juices on infection by Escherichia coli enteroaggregative in larvae of Tenebrio molitor. (A) Preventive Trial; (B) Treatment trial.

Phosphate-Saline Buffer - PBS, Enteroagregative *Escherichia Coli* EAEC, *Plantonia insignis* – Pij, *Lacticaseibacillus rhamnosus* – Lr.

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ANEXOS

SCIENTIFIC REPORTS
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Schinus terebinthifolia leaf lectin (SteLL) has anti-infective action and modulates the response of *Staphylococcus aureus*-infected macrophages

Isana Maria de Souza Feitosa Lima[1], Adrielle Zagmignan[1], Deivid Martins Santos[1], Hermerson Sousa Maia[1], Lucas dos Santos Silva[1], Brenda da Silva Cutrim[1], Silvamara Leite Vieira[1], Clovis Macêdo Bezerra Filho[2], Eduardo Martins de Sousa[1], Thiago Henrique Napoleão[2], Karen Angeliki Krogfelt[3,4], Anders Løbner-Olesen[5], Patrícia Maria Guedes Paiva[2] & Luís Cláudio Nascimento da Silva[1*]

Staphylococcus aureus **is recognized as an important pathogen causing a wide spectrum of diseases. Here we examined the antimicrobial effects of the lectin isolated from leaves of *Schinus terebinthifolia* Raddi (SteLL) against *S. aureus* using *in vitro* assays and an infection model based on *Galleria mellonella* larvae. The actions of SteLL on mice macrophages and *S. aureus*-infected macrophages were also evaluated. SteLL at 16 µg/mL (8 × MIC) increased cell mass and DNA content of *S. aureus* in relation to untreated bacteria, suggesting that SteLL impairs cell division. Unlike ciprofloxacin, SteLL did not induce the expression of *recA*, crucial for DNA repair through SOS response. The antimicrobial action of SteLL was partially inhibited by 50 mM *N*-acetylglucosamine. SteLL reduced staphyloxathin production and increased ciprofloxacin activity towards *S. aureus*. This lectin also improved the survival of *G. mellonella* larvae infected with *S. aureus*. Furthermore, SteLL induced the release of cytokines (IL-6, IL-10, IL-17A, and TNF-α), nitric oxide and superoxide anion by macrophagens. The lectin improved the bactericidal action of macrophages towards *S. aureus*; while the expression of IL-17A and IFN-γ was downregulated in infected macrophages. These evidences suggest SteLL as important lead molecule in the development of anti-infective agents against *S. aureus*.**

Staphylococcus aureus is recognized as an important pathogen causing a wide spectrum of diseases including cutaneous and blood stream infections[1,2]. This versatility is ensured by the high ability of this microorganism to acquire drug resistance and to produce virulence factors that are regulated by complex genetic networks[2–5]. The multiple virulence factors identified in *S. aureus* play different roles during the infection such as adhesion, host lesions and evasion of the immune system, even from professional phagocytes such as macrophages[6–8].

It has been reported that the dissemination of *S. aureus* is associated to the capacity of this bacterium to survive and replicate inside the phagocytes (in both phagosome and/or cytoplasm), and modulate important cellular mechanisms such as autophagy, apoptosis and pyronecrosis[9–11]. *S. aureus* is also able to release several effector molecules to suppress or enhance cytokine production (including IL-1β, IL-17 and TNF) as well as to damage immune cells and host tissues[6,12–14]. Thus, compounds able to improve and/or regulate the cellular immune response have been pointed out as promising lead drugs for treatment of microbial infections and also to improve the general understanding related to host-pathogen interactions[15–19].

[1]Programas de Pós-Graduação, Universidade Ceuma, São Luís, Maranhão, Brazil. [2]Departamento de Bioquímica, Universidade Federal de Pernambuco, Recife, Pernambuco, Brazil. [3]Department of Viral and Microbial Diagnostics, Statens Serum Institut, Copenhagen, Denmark. [4]Department of Science and Environment, Roskilde University, 4000, Roskilde, Denmark. [5]Department of Biology, Section for Functional Genomics, University of Copenhagen, Copenhagen, Denmark. *email: luiscn.silva@ceuma.br

Hindawi
Advances in Pharmacological and Pharmaceutical Sciences
Volume 2020, Article ID 1258707, 11 pages
https://doi.org/10.1155/2020/1258707

Research Article

Antioxidant Action and *In Vivo* Anti-Inflammatory and Antinociceptive Activities of *Myrciaria floribunda* Fruit Peels: Possible Involvement of Opioidergic System

Izabelly Bianca da Silva Santos,[1] Bruno Santos dos Santos,[1] João Ricardhis Saturnino de Oliveira,[1] Wêndeo Kennedy Costa,[1] Adrielle Zagmignan,[2] Luís Cláudio Nascimento da Silva,[2] Magda Rhayanny Assunção Ferreira,[3] Vilmar Luiz Lermen,[4] Maria Silvanete Benedito de Sousa Lermen,[4] Alexandre Gomes da Silva,[1] Rafael Matos Ximenes,[5] Luiz Alberto Lira Soares,[3] Patrícia Maria Guedes Paiva,[1] Vera Lúcia de Menezes Lima,[1] Maria Tereza dos Santos Correia,[1] and Márcia Vanusa da Silva[1]

[1]*Departamento de Bioquímica, Universidade Federal de Pernambuco, Recife, PE 50670-901, Brazil*
[2]*Programa de Pós-Graduação, Universidade Ceuma, São Luís, MA 65075-120, Brazil*
[3]*Departamento de Ciências Farmacêuticas, Universidade Federal de Pernambuco, Recife, PE 50670-901, Brazil*
[4]*Comunidade Serra dos Paus Dóias Chapada do Araripe, Exu, PE 56230-000, Brazil*
[5]*Departamento de Antibióticos, Universidade Federal de Pernambuco, Recife, PE 50670-901, Brazil*

Correspondence should be addressed to Márcia Vanusa da Silva; marciavanusa@yahoo.com.br

Received 16 November 2019; Revised 21 February 2020; Accepted 13 March 2020; Published 27 April 2020

Academic Editor: Srinivas Mutalik

This work evaluated the antioxidant properties and *in vivo* antinociceptive and anti-inflammatory effects of extracts obtained from fruit peels of *Myrciaria floribunda* (H. West ex Willd.) O. Berg (Myrtaceae). This plant is popularly known in Brazil as *Cambuí* or *camboim*. Different extracts were submitted to comparative analysis to determine the content of selected phytochemical classes (levels of total phenols, flavonoids, and monomeric anthocyanins) and the *in vitro* antioxidant potentials. The extract with higher potential was selected for *in vivo* evaluation of its antinociceptive and anti-inflammatory action. Finally, the chemical characterization of this extract was performed by high-performance liquid chromatography (HPLC). MfAE (extract obtained using acetone as solvent) showed the higher levels of phenols (296 mg GAE/g) and anthocyanins contents (35.65 mg Cy-3-glcE/g) that were associated with higher antioxidant activity. MfAE also exhibited *in vivo* anti-inflammatory and analgesic propertiers. This fraction inhibited the inflammatory and neurogenic phases of pain, and this effect was reversed by naloxone (suggesting the involvement of opioidergic system). MfAE reduced the abdominal contortions induced by acetic acid. The HPLC analysis revealed the presence of gallic acid (and its derivatives) and ellagic acid. Taken together, these data support the use of *M. floribunda* fruit peels for development of functional foods and nutraceutics.

1. Introduction

Free radicals (and associated reactive species) are in general derived from normal metabolism and are crucial for redox signaling pathways and immune defense [1–4]. However, these molecules can interact with cellular structures leading to impairment of physiological systems [5–7]. In this sense, the oxidative stress has been associated with the etiology of several pathologies such as inflammatory disorders, chronic pain, and degenerative diseases [8–10].

Inflammation is a complex condition triggered by several stimuli (mechanical injuries, toxic compounds, tissue

CAPÍTULO 9

Lactobacillus fermentum: POTENCIAL BIOTECNOLOGICO PARA APLICAÇÕES NA INDÚSTRIA FARMACÊUTICA E ALIMENTÍCIA

Data de aceite: 10/12/2019

Brenda Ferreira de Oliveira
Universidade CEUMA, Curso de Biomedicina.
São Luís, Maranhão.

Amanda Caroline de Souza Sales
Universidade CEUMA, Curso de Biomedicina.
São Luís, Maranhão.

Daniele de Aguiar Moreira
Universidade CEUMA, Curso de Biomedicina.
São Luís, Maranhão.

Mari Silma Maia da Silva
Universidade CEUMA, Programa de Pós-graduação em Biologia Microbiana. São Luís, Maranhão.

Gabrielle Damasceno Evangelista Costa
Universidade CEUMA, Programa de Pós-graduação em Biologia Microbiana. São Luís, Maranhão.

Gustavo Henrique Rodrigues Vale de Macedo
Universidade CEUMA, Programa de Pós-graduação em Biologia Microbiana. São Luís, Maranhão.

Lívia Muritiba Pereira de Lima Coimbra
Universidade CEUMA, Curso de Nutrição e Programa de Pós-graduação em Biodiversidade e Biotecnologia (REDE BIONORTE).
São Luís, Maranhão.

Rita de Cássia Mendonça de Miranda
Universidade CEUMA, Programa de Pós-graduação em Meio Ambiente, Programa de Pós-graduação em Biologia Microbiana e Programa de Pós-graduação em Biodiversidade e Biotecnologia (REDE BIONORTE).
São Luís, Maranhão.

Adrielle Zagmignan
Universidade CEUMA, Curso de Nutrição e Programa de Pós-graduação em Biodiversidade e Biotecnologia (REDE BIONORTE).
São Luís, Maranhão.

Luís Cláudio Nascimento da Silva
Universidade CEUMA; Curso de Biomedicina, Programa de Pós-graduação em Biologia Microbiana, Programa de Pós-graduação em Odontologia e Programa de Pós-graduação em Biodiversidade e Biotecnologia (REDE BIONORTE).
São Luís, Maranhão.

RESUMO: Probióticos são definidos como suplementos alimentares à base de microrganismos vivos que promovem benefícios através da interação com a microbiota intestinal. Estas preparações atuam diretamente na resistência a infecções, no melhor aproveitamento de vitaminas e também estão envolvidos na fisiopatologia de diversas doenças. *Lactobacillus fermentum* é uma bactéria frequentemente utilizada por apresentar características desejáveis, como alta tolerância ao pH e a sais biliares, capacidade

CAPÍTULO 10

Lactobacillus rhamnosus E O DESENVOLVIMENTO DE PRODUTOS BIOATIVOS

Data de aceite: 10/12/2019

Amanda Caroline de Souza Sales
Universidade CEUMA, Curso de Biomedicina.
São Luís, Maranhão.

Brenda Ferreira de Oliveira
Universidade CEUMA, Curso de Biomedicina.
São Luís, Maranhão.

Deivid Martins Santos
Universidade CEUMA, Curso de Biomedicina.
São Luís, Maranhão.

Mari Silma Maia da Silva
Universidade CEUMA, Programa de Pós-graduação em Biologia Microbiana. São Luís, Maranhão.

Gabrielle Damasceno Evangelista Costa
Universidade CEUMA, Programa de Pós-graduação em Biologia Microbiana. São Luís, Maranhão.

Gustavo Henrique Rodrigues Vale de Macedo
Universidade CEUMA, Programa de Pós-graduação em Biologia Microbiana. São Luís, Maranhão.

Lívia Muritiba Pereira de Lima Coimbra
Universidade CEUMA, Curso de Nutrição e Programa de Pós-graduação em Biodiversidade e Biotecnologia (REDE BIONORTE).
São Luís, Maranhão.

Rita de Cássia Mendonça de Miranda
Universidade CEUMA, Programa de Pós-graduação em Meio Ambiente, Programa de Pós-graduação em Biologia Microbiana e Programa de Pós-graduação em Biodiversidade e Biotecnologia (REDE BIONORTE).
São Luís, Maranhão.

Adrielle Zagmignan
Universidade CEUMA, Curso de Nutrição e Programa de Pós-graduação em Biodiversidade e Biotecnologia (REDE BIONORTE).
São Luís, Maranhão.

Luís Cláudio Nascimento da Silva
Universidade CEUMA; Curso de Biomedicina, Programa de Pós-graduação em Biologia Microbiana, Programa de Pós-graduação em Odontologia e Programa de Pós-graduação em Biodiversidade e Biotecnologia (REDE BIONORTE).
São Luís, Maranhão.

RESUMO: Os probióticos fazem parte dos chamados alimentos funcionais e são definidos como microrganismos vivos não patogênicos que, quando consumidos, podem trazer efeitos benéficos a saúde do hospedeiro, prevenindo ou tratando determinadas doenças, como infecções bacterianas. A inserção de, principalmente, bactérias ácido-láticas em bebidas e alimentos é uma tendência global, e as qualidades funcionais desses produtos vêm sendo cientificamente demonstradas. O presente estudo teve como objetivo discutir o