Universidade Federal do Maranhão

Centro de Ciências Biológicas e da Saúde

Programa de Pós-Graduação em Ciências da Saúde

**Doutorado**

# PROSPECÇÃO DE MICRORGANISMOS COM POTENCIAL PROBIÓTICO CONTRA ENTEROPATÓGENOS BACTERIANOS

MONIQUE SANTOS DO CARMO

São Luís

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Tese apresentada ao Programa de Pós-Graduação em Ciências da Saúde da Universidade Federal do Maranhão, como requisito parcial para obtenção do título de Doutora em Ciências da Saúde.

Orientador: Prof. Dr. Valério Monteiro Neto
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"Conócete. Acetate. Supérate"
Conhece-te. Aceita-te. Supera-te.
Santo Agostinho

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# SUMÁRIO

# LISTA DE ABREVIATURAS E SIGLAS

| | |
|---|---|
| DAA | Diarreia associada ao uso de antibióticos |
| DEC | *Escherichia coli* diarreiogênica |
| DII | Doença inflamatória intestinal |
| EAEC | *Escherichia coli* enteroagregativa |
| EIEC | *Escherichia coli* enteroinvasora |
| EPEC | *Escherichia coli* enteropatogênica |
| ETEC | *Escherichia coli* enterotoxigênica |
| GI | Gastrointestinal |
| LPS | Lipopolissacarídeo |
| MRSA | *Staphylococcus aureus* resistente à meticilina |
| STEC | *Escherichia coli* produtora da toxina de Shiga |
| TGI | Trato gastrointestinal |
| TNF-α | Fator de necrose tumoral alfa |
| OMS | Organização Mundial da Saúde |
| pH | Potencial hidrogeniônico |
| UFC | Unidades formadoras de colônias |

## RESUMO

A diarreia bacteriana é uma enfermidade infecciosa que ocorre com significativa morbidade e mortalidade em todo o mundo, principalmente em países subdesenvolvidos e em desenvolvimento. Os protocolos recomendados para tratamento incluem a reposição hidroeletrolítica, eventualmente associada a antimicrobianos. Entretanto, tais medidas não são suficientes para eliminar todos os efeitos deletérios desse processo infeccioso, tais como a redução do número de evacuações e a reposição da microbiota intestinal. Medidas mais racionais poderiam incluir abordagens que pudessem auxiliar na prevenção e/ou tratamento infecção intestinal, sem acentuar o desequilíbrio da microbiota e sem exercer pressões seletivas que poderiam propiciar a emergência de microrganismos resistentes. Uma das possibilidades consiste no uso de probióticos, no entanto, diferentes estudos têm demonstrado que os efeitos benéficos desses microrganismos podem ser espécie-específicos ou, até mesmo, linhagem-específicos. Desta forma, nem todos os probióticos apresentam atividades contra as bactérias enteropatogênicas comumente associadas à doença diarreica. Portanto, o objetivo desse estudo foi realizar a prospecção de microrganismos com potencial probiótico em fezes de crianças contra enteropatógenos bacterianos de relevância clínica. Culturas microbiológicas de fezes foram realizadas em meios de cultura seletivos para *Bifidobacterium* e *Lactobacillus.* As colônias típicas de bacilos gram-positivos e catalase-negativos foram submetidas aos ensaios de tolerância a sais biliares, pH ácido e lisozima e capacidade de ligação à mucina. Os isolados com perfil probióticos foram analisadas com relação à atividade antimicrobiana contra enteropatógenos e também para a atividade inibitória da adesão à mucina. Posteriormente, as bactérias foram identificadas pelo sequenciamento do rRNA 16S. Foi produzido um artigo de revisão sobre os principais mecanismos de ação dos probióticos e suas perspectivas clínicas para tratamento e prevenção da diarreia em crianças. Adicionalmente, foi elaborado um artigo resultante da investigação do potencial probiótico dos isolados de *Bidifidobacterium bifidum* A14.2, *Bidifidobacterium longum* subsp. *longum* C25.3, *B. longum* N49.3, *Bidifidobacterium animalis* subsp. *lactis* L56.1 e *Lactobacillus fermentum* L54.2, uma vez que foram resistentes ao pH ácido, sais biliares, lisozima e apresentaram ligação à mucina. Além disso, inibiram a adesão e o crescimento de *Escherichia coli* enteroagregativa 042 (EAEC 042), *E. coli* enteropatogênica e-2348/69 (EPEC e-2348/69), *E.* coli enterohemorrágica EDL 933 (EHEC EDL 933), *E. coli* enterotoxigênica 1661-1 (ETEC 1661-1), *Salmonella* Choleraesuis INCQS 028 e *Shigella flexneri* 2a. Os dados obtidos são promissores e fornecem subsídios para o futuro uso desses potenciais probióticos em ensaios com animais visando confirmar a eficácia das mesmas em modelos de infecção experimental.


Palavras-chave: probióticos; *Lactobacillus*; *Bifidobacterium*; enteropatógenos bacterianos; diarreia.

## ABSTRACT

Bacterial diarrhea is an infectious disease that occurs with significant morbidity and mortality worldwide, principally in underdeveloped and developing countries. The recommended protocols for treatment include hydro-electrolyte replacement, eventually associated with antimicrobials. However, such measures are not sufficient to eliminate all deleterious effects of this infectious process, such as reduction in the number of evacuations and restoration of the intestinal microbiota. More rational measures could include approaches that can aid in the prevention and/or treatment of intestinal infection, without increasing the imbalance of the microbiota and without exerting selective pressures that could lead to the emergence of resistant microorganisms. One possibility consists in the use of probiotics, however, different studies have shown that many beneficial effects of these microorganisms can be species-specific or even strain-specific. Thus, not all probiotics may be active against enteropathogenic bacteria commonly associated with diarrheal disease. Therefore, the objective of this study was to prospect for microorganisms in newborns' feces with probiotic potential against prevalent bacterial enteropathogens. Stool microbiological cultures were performed in selective culture media for *Bifidobacterium* and *Lactobacillus*. Typical gram-positive and catalase-negative bacilli colonies were submitted to bile salt tolerance, acid pH and lysozyme tests and binding ability to mucin. The bacterial isolates with probiotic profile were analyzed for antimicrobial activity against enteropathogenic bacteria and also for the inhibitory activity of adhesion to mucin. Subsequently, the bacteria were identified by sequencing the 16S rRNA. It was produced a review article on the main probiotic mechanisms of action and clinical perspectives for treatment and prevention of diarrhea in children. Additionally, an article resulting from the investigation of the probiotic potential of the isolates of *Bidifidobacterium bifidum* 14.2, *Bidifidobacterium longum* subsp. *longum* 25.3, B. *longum* 49.3, *Bidifidobacterium animalis* subsp. *lactis* 56.1 and *Lactobacillus fermentum* 54.2, as they were resistant to acid pH, bile salts, lysozyme and showed mucin binding was written. Additionally, these strains inhibited binding to mucin and the growth of enteropathogens, such as: enteroaggregative *Escherichia coli* 042 (EAEC 042), enteropathogenic *E. coli* e2348/69 (EPEC e2348/69), enterohemorrhagic *E. coli* 933 EDL (EHEC 933 EDL), enterotoxigenic *E. coli* 1661-1 (ETEC 1661-1), *Salmonella* Choleraesuis INCQS 028, and *Shigella flexneri* 2a. The data obtained are promising and provide supports for the future use of these potential probiotics in animal tests to confirm their efficacy in experimental infection models.


Keywords: Probiotics; *Lactobacillus*; *Bifidobacterium*; Bacterial enteropathogens; Diarrhea.

## 1 INTRODUÇÃO

Segundo as estimativas oficiais (UNICEF, 2018), a diarreia é apontada como a segunda maior causa de morte entre crianças menores de cinco anos de idade e que pode deixar sequelas relacionadas ao comprometimento no desenvolvimento físico e cognitivo. Em contrapartida, a antibioticoterapia não é recomendada pela possibilidade do paciente continuar a albergar o patógeno bacteriano, por não ser eficaz nos casos da diarreia viral e propiciar uma piora do quadro clínico do paciente, além de não sanar todos os efeitos deletérios desse processo infeccioso.

Neste contexto, os probióticos surgem como uma abordagem terapêutica e de prevenção inovadora, que pode ser empregada na forma de medicamentos liofilizados/suspensões ou suplementos alimentares, os quais irão estimular a proliferação de bactérias benéficas em detrimento de microrganismos enteropatogênicos (PUEBLA-BARRAGAN;REID, 2019). Contudo, o processo de triagem de potenciais probióticos passa pela análise de certos pré-requisitos relacionados à capacidade do microrganismo resistir ao pH ácido, tolerar enzimas gástricas e intestinais, secretar compostos com atividade antimicrobiana, aderir ao muco e epitélio intestinal e inibir a adesão de enteropatógenos, reduzindo ou eliminando suas populações. Todos esses ensaios têm sido realizados com isolados de amostras de leite, fezes (humanas ou de animais) e do meio ambiente como padrão-ouro na prospecção de probióticos.

O intestino é o sítio com a maior diversidade e complexidade de microrganismos aeróbios e anaeróbios do corpo humano. Portanto, a seleção de probióticos a partir da microbiota intestinal humana representa uma importante estratégia preventiva e terapêutica, principalmente para o tratamento de uma doença com elevados índices de morbidade e mortalidade. A identificação de novas espécies a partir de pesquisas nacionais pode auxiliar na redução dos custos e para o progresso científico e tecnológico do Brasil. Adicionalmente, uma das grandes preocupações da Organização Mundial da Saúde é a implementação de novas terapias que não atuem como pressão seletiva, ao propiciar a seleção de patógenos cada vez mais resistentes aos antibióticos.

Dessa forma, diante da necessidade de novas alternativas para o tratamento das infecções intestinais, a prospecção de novos microrganismos com potencial probiótico pode representar um avanço importante para a elevação do nível de saúde entre a população infantil, seja em países subdesenvolvidos ou em desenvolvimento.

Dentre as bactérias consideradas probióticas, as do gênero *Bifidobacterium* e *Lactobacillus* tem sido as mais estudadas, principalmente porque muitas espécies fazem parte da microbiota intestinal normal. Apesar das evidências pontuadas na literatura, são necessários mais estudos que possam explorar a diversidade desses gêneros e evidenciar novas cepas que atuem na regressão ou atenuação da diarreia e até mesmo de outros quadros infecciosos.

A tese foi produzida no formato de artigo, sendo composta por um de revisão (publicado na revista *Food & Function* – Qualis B1 em Medicina I) e outro no formato de artigo original (submetido à revista Anaerobe – Qualis B1 em Medicina I).

# 2 REFERENCIAL TEÓRICO

## 2.1 DOENÇA DIARREICA

Segundo a Organização Mundial de Saúde, a doença diarreica apresenta prevalência elevada em todo o mundo, sendo uma causa importante de mortalidade infantil (WHO, 2017). Estima-se que 480.000 crianças, com menos de cinco anos, morrem por ano em consequência da doença diarreica (UNICEF, 2018). No geral, as crianças mais frequentemente acometidas residem nas regiões menos desenvolvidas do mundo, com destaque para Índia, Nigéria, Afeganistão, Paquistão e Etiópia (WHO, 2017). Segundo o último censo do Ministério da Saúde, foram registrados mais de 5.000.000 de casos da diarreia aguda no Brasil (BRASIL, 2018).

Esses dados demonstram a importância dessa enfermidade para a saúde pública, uma vez que causa profundos impactos sobre as taxas de morbidade e mortalidade infantil (BRYCE et al., 2005; BANAJEH, 2017; HAY, 2017). Altas taxas de morbidade por doença diarreica são preocupantes porque a diarreia infantil pode deixar sequelas que se manifestarão em longo prazo sobre o desenvolvimento físico e cognitivo da criança (BLACK;BROWN;BECKER, 1984; GUERRANT et al., 1999; DERBY et al., 2014; LAMBERTI et al., 2014; TATE et al., 2016).

A diarreia é definida pela presença de três ou mais episódios de fezes líquidas ou amolecidas ao dia, sendo acompanhada ou não por febre e vômitos. Do ponto de vista clínico, apresenta-se sob três formas: 1-diarreia aquosa aguda: possui durabilidade de várias horas ou dias; 2-diarreia sanguinolenta: também conhecida como disenteria, pode apresentar muco além de sangue; 3-diarreia persistente: possui durabilidade de 14 dias ou mais (WHO, 2017).

Várias bactérias, vírus e parasitas intestinais estão associados à infecção no ser humano, que são transmitidos pela via oral-fecal (ELLIOTT, 2007). Isso geralmente ocorre por causa das precárias condições higiênico-sanitárias dos países subdesenvolvidos e em desenvolvimento, como é o caso do Brasil. Segundo a Organização Mundial da Saúde, aproximadamente 2,5 bilhões de pessoas não tem acesso às instalações sanitárias básicas e 780 milhões não tem acesso à água potável (WHO, 2017). Tais condições contribuem para a disseminação desses patógenos e a prevalência da diarreia.

Entre os principais agentes da diarreia bacteriana estão os diferentes sorotipos de *Salmonella*, *Shigella* spp. e as categorias de *E. coli* diarreiogênicas (DEC). Contudo, as DEC

estão entre as causas predominantes de doença diarreica em nível mundial, principalmente na infância (PARASHAR et al., 2006; AL-GALLAS et al., 2007; BUERIS et al., 2007). Essas bactérias estão distribuídas em seis categorias principais: *E. coli* enteroagregativa (EAEC), *E. coli* produtora de toxina Shiga (STEC), *E. coli* enteroinvasiva (EIEC), *E. coli* enteropatogênica (EPEC), *E. coli* enterotoxigênica (ETEC) e *E.coli* aderente-invasiva (AIEC). Essa classificação tem por base as características epidemiológicas e clínicas, fatores de virulência e sorotipos (NATARO;KAPER, 1998). Eventualmente, a doença diarreica pode ser desencadeada por outras bactérias, tais como: *Aeromonas* spp., *Vibrio* spp., *Campylobacter* spp., *Clostridium* spp. e *Plesiomonas* spp. (ELLIOTT, 2007; KOTLOFF et al., 2013).

Embora muitos dos casos de diarreia sejam ocasionados por vírus, um elevado número de episódios de diarreia bacteriana tem sido relatado. Um estudo desenvolvido na Índia demonstrou uma redução de 10,5 % dos episódios de diarreia viral em função de um incremento de 6,3% da diarreia causada por *E. coli* (MELLOR et al., 2016). Concomitantemente, infecções intestinais causadas por *Salmonella* e *Shigella* spp. foram prevalentes no sul do Vietnã (ANDERS et al., 2015). Em áreas suburbanas de Cartum (Sudão), 48% dos casos de diarreia aguda em crianças menores de cinco anos estavam relacionados com as DEC (SAEED;ABD;SANDSTROM, 2015). Por último, em um hospital infantil dos Estados Unidos, constatou-se uma maior prevalência da diarreia bacteriana comparada com a viral (STOCKMANN et al., 2016).

Convêm destacar que a doença diarreica pode ocorrer também por causa do uso prolongado ou inadequado de antimicrobianos, culminando no quadro denominado diarreia associada ao uso de antibióticos (DAA). Neste caso, o microrganismo *Clostridium difficile* é um dos agentes etiológicos mais frequentes (MADA;ALAM, 2017). Além disso, *Sthaphylococcus aureus* resistente à meticilina (MRSA) tem sido descrita como um patógeno importante na DAA (BOYCE;HAVILL, 2005; LO;BORCHARDT, 2009).

A gravidade e a prevalência das infecções pelas DEC estão bem documentadas em países em desenvolvimento, sendo que o seu tratamento envolve as mesmas medidas de manejo indicadas para a doença diarreica por outras bactérias, as quais incluem um tratamento de suporte dependendo da presença de sinais de desidratação e a prescrição de antibióticos ou de antiparasitários apenas para alguns casos particulares (GUERRANT et al., 2001; ELLIOTT, 2007).

Nos casos de infecções parasitárias (como amebíase e giardíase) e de infecções bacterianas sistêmicas, da shigelose e da cólera, a quimioterapia antimicrobiana adequada pode reduzir o curso clínico da doença e a duração da eliminação fecal do agente causador (GUERRANT et al., 2001; SHANE et al., 2017). Entretanto, quando a terapia empírica é realizada com antibióticos de largo espectro ou quando o tratamento falha em consequência da resistência à droga empregada, ela pode possibilitar a disseminação da resistência a outras bactérias (HINES;NACHAMKIN, 1996; GUERRANT et al., 2001; SHANE et al., 2017). Além disso, a terapia empírica pode resultar no uso desnecessário de antibióticos, tendo em vista a elevada prevalência de infecções virais (BON et al., 1999; PARASHAR et al., 2003; PARASHAR et al., 2006; BALKAN;ÇELEBI, 2017; GODDARD, 2017).

Diante desse contexto, são necessárias alternativas que possam amenizar ou conter os quadros de diarreia infecciosa bacteriana. Entre essas medidas, a comunidade científica tem investido no estudo dos probióticos, uma vez que são microrganismos que podem conferir vários benefícios ao hospedeiro, incluindo a reestabelecimento da microbiota, redução do número de evacuações e de internações, impactando diretamente na saúde dos indivíduos e na economia dos países.

## 2.2 PROBIÓTICOS

Diante da complexidade estrutural e funcional da microbiota do trato gastrointestinal (TGI), as propriedades benéficas de alguns microrganismos têm sido avaliadas no sentido de tentar implementar uma terapia alternativa para a prevenção e tratamento de muitas enfermidades infecciosas. Os primeiros estudos foram conduzidos pelo cientista russo Ilya Ilyich Metchnikoff no início do século XX que demonstrou os efeitos benéficos de um alimento fermentado para o metabolismo humano (METCHNIKOFF, 1907; METCHNIKOFF;MITCHELL, 1907). Tal descoberta rendeu ao biólogo pesquisador o Prêmio Nobel de Medicina no ano de 1908.

No ano de 1965, Lilly e Stillwell descobriram que determinadas “substâncias” ou “fatores” secretados pelo protista ciliado *Colpidium campylum* eram capazes de servir como suporte para o crescimento de outro da espécie *Tetrahymena pyriformis*. Sendo assim, os pesquisadores desenvolveram o primeiro conceito de probióticos que deriva do grego e significa “para a vida” (LILLY;STILLWELL, 1965).

Atualmente, o conceito adotado no meio científico é o da Organização Mundial da Saúde (OMS) e Organização das Nações Unidas para Alimentação e Agricultura (FAO) que define o probiótico como qualquer microrganismo vivo que, quando administrado em quantidades adequadas pode conferir uma série de benefícios para o hospedeiro (GROUP, 2001). Dentro dessa definição, os microrganismos da microbiota intestinal não são considerados probióticos até o momento em que forem isolados, purificados e analisados quanto às suas propriedades benéficas (KLEEREBEZEM;VAUGHAN, 2009).

Entre os principais benefícios para a saúde humana, destacam-se a redução da duração da diarreia e do tempo de hospitalização (VANDENPLAS, 2016), alívio dos sintomas relacionados à intolerância da lactose (ALMEIDA et al., 2012; VONK et al., 2012; PAKDAMAN et al., 2016), alergias (OUWEHAND et al., 2009; DEL GIUDICE et al., 2017; DENNIS-WALL et al., 2017), atenuação dos níveis de colesterol sérico (LIU et al., 2017; WANG et al., 2017), prevenção do câncer (DJALDETTI;BESSLER, 2017; KAHOULI et al., 2017; LI et al., 2017; SABER et al., 2017) e diabetes (HU et al., 2017; WANG et al., 2017).

Apesar dos efeitos benéficos, alguns probióticos podem causar efeitos adversos em alguns pacientes (portadores da doença de Crohn, colite ulcerativa), tais como náuseas/vômitos, dor epigástrica e constipação (ROLFE et al., 2006; GOLDENBERG et al., 2017).

Dentre os principais grupos microbianos estudados como probióticos, destacam-se os gêneros *Lactobacillus*, *Bifidobacterium*, *Saccharomyces, Streptococcus*, *Escherichia*, *Lactococcus* e *Enterococcus*, sendo estes dois últimos em menor escala (DE VRESE;SCHREZENMEIR, 2008; AMER et al., 2017) (Tabela 1).

**Tabela 1**. Principais espécies bacterianas estudadas como probióticos.

| GÊNEROS | ESPÉCIES | EFEITOS COMPROVADOS | REFERÊNCIAS |
|---|---|---|---|
| ***Bacillus*** | *clausii* | Produção de metabólitos contra *S. aureus*, *E. faecium* e *C. difficile*; Estimulação da secreção de IFN-γ e da proliferação de células $TCD4^+$ | URDACI;BRESSOLLIER;PINCHUK, 2004 |
| | | Redução dos efeitos colaterais da antibioticoterapia para *H.pylori* | NISTA et al., 2004 |
| | *coagulans* | Redução dos movimentos peristálticos, flatulência e dor abdominal em pacientes com síndrome do intestino irritável (SII); | DOLIN, 2009; HUN, 2009 |
| ***Bifidobacterium*** | *adolescentis* | Ativação de macrófagos, aumento da produção de TNF-α e NO em modelo de células de câncer de cólon | LEE et al., 2008 |
| | | Prevenção da enterocolite necrotizante | WU et al., 2017 |
| | | Inibição da adesão de rotavírus em células MA104 | FERNANDEZ-DUARTE et al., 2017 |
| | *animalis* | Redução do tempo de trânsito colônico em mulheres saudáveis | MARTEAU et al., 2002 |
| | | Proteção contra a inflamação causada por ETEC K88 em células intestinais Caco-2 | ROSELLI et al., 2006 |
| | *bifidum* | Redução da incidência da diarreia aguda em crianças | SAAVEDRA et al., 1994 |
| | | Estimulação do crescimento de *Bifidobacterium* spp. em idosos | BARTOSCH et al., 2005 |
| | | Inibição de *H. pylori in vitro* e *in vivo* | CHENOLL et al., 2011 |
| | | Melhora do quadro de gastroenterite causada por rotavírus por estimular a secreção de fatores de proteção de mucosa | KAWAHARA et al., 2017 |
| | *infantis* | Inibição de *Salmonella in vitro* | RAHIMIFARD;NASERI, 2016 |

| | | | |
|---|---|---|---|
| | | Redução da incidência da enterocolite necrotizante em neonatos em uma Unidade de Terapia Intensiva | HOYOS, 1999 |
| | | Melhora da estabilidade do epitélio intestinal | EWASCHUK et al., 2008 |
| | | Redução da incidência da enterocolite necrotizante | HOYOS, 1999 |
| | *longum* | Atividade anti-tumoral via modulação de biomarcadores do câncer de cólon | SINGH et al., 1997 |
| | | Inibição da infecção por rotavírus *in vitro* | MUÑOZ et al., 2011 |
| | | Melhora do quadro de colite em camundongos via inibição da ativação de macrófagos e restauração do balanço Th17/Treg | LIM et al., 2016 |
| ***Enterococcus*** | *faecium* | Redução dos níveis de colesterol sérico | HLIVAK et al., 2005 |
| | | Proteção do epitélio intestinal e atenuação da secreção de IL-8 induzida por ETEC em enterócitos | TIAN et al., 2016 |
| | | Inibição de *C.difficile in vivo* | MANSOUR et al., 2017 |
| | | Redução da secreção de citocinas pró-inflamatórias em modelo de infecção com ETEC | KERN et al., 2017 |
| ***Escherichia*** | *coli* | Regressão do quadro de colite ulcerativa | KRUIS et al., 2004 |
| | | Reparo do epitélio intestinal | ZYREK et al., 2007 |
| | | Estimulação da secreção de uma ß-defensina humana | SCHLEE et al., 2007 |
| | | Inibição da adesão e invasão de AIEC em cultura de células intestinais da linhagem 407 | BOUDEAU et al., 2003 |
| | | Redução da dor abdominal em pacientes com SII | SINN et al., 2008 |
| | | Inibição da invasão do vírus H9N2 em células dendríticas pela expressão da proteína de camada S | GAO et al., 2016 |

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| ***Lactobacillus*** | *brevis* | Melhora do quadro de colite em camundongos via inibição da ativação de macrófagos e restauração do balanço Th17/Treg | LIM et al., 2016 |
| | *casei* | Inibição da adesão de rotavírus em células MA104 | FERNANDEZ-DUARTE et al., 2017 |
| | | Redução da severidade da constipação e melhora da consistência das fezes em pacientes com quadro de constipação crônica | KOEBNICK et al., 2003 |
| | | Inibição *in vitro* e *in vivo* de *H. pylori* | SGOURAS et al., 2004 |
| | | Redução do peso e melhora do metabolismo lipídico em crianças obesas | NAGATA et al., 2017 |
| | *delbrueckii* | Inibição da adesão e dos efeitos citotóxicos de *C. difficile* em células Caco-2 | BANERJEE;MERKEL;BHUNIA, 2009 |
| | *fermentum* | Redução da incidência de infecções gastrointestinais e do trato respiratório superior em crianças | MALDONADO et al., 2012 |
| | | Inibição da infecção por *S. aureus* desencadeada por implantes cirúrgicos em camundongos | GAN et al., 2002 |
| | | Inibição de *E. coli* e *G. vaginalis* em *in vitro* e *in vivo* | KAEWNOPPARAT et al., 2013 |
| | | Inibição de *Candida* spp. *in vitro* | DO CARMO et al., 2016 |
| | *plantarum* | Redução dos níveis de colesterol sérico | NGUYEN;KANG;LEE, 2007 |
| | | Redução da dor abdominal e frequência de evacuações em pacientes com SII | NIEDZIELIN;KORDECKI;ENA BIRKENFELD, 2001 |
| | | Efeito protetor para o epitélio intestinal e regulação das proteínas das "*tight junctions"* | ANDERSON et al., 2010; KARCZEWSKI et al., 2010 |
| | | Prevenção da patogênese severa em ratos infectados com *Leptospira interrogans* | POTULA et al., 2017 |

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|---|---|---|---|
| | *rhamnosus* | Redução da duração da diarreia e do tempo de hospitalização em crianças | GUANDALINI et al., 2000; KORPELA et al., 2016 |
| ***Saccharomyces*** | *boulardii* | Redução da duração da diarreia e do tempo de hospitalização em crianças | VILLARRUEL et al., 2007; DAS;GUPTA;DAS, 2016 |
| ***Streptococcus*** | *thermophilus* | Redução da incidência da diarreia aguda em crianças | SAAVEDRA et al., 1994 |

Após a descoberta dos microrganismos com propriedades probióticas, houve uma grande expansão dos produtos suplementados com esses estirpes, dentre os quais destacam-se o iogurte, leite fermentado e não-fermentado, queijo, sucos, *smoothies*, barras de cereais, dentre outras fórmulas infantis. Adicionalmente, os probióticos são comercializados como suplementos dietéticos e medicamentos (CHAMPAGNE;GARDNER;ROY, 2005; FOLIGNÉ;DANIEL;POT, 2013; PASSARIELLO;AGRICOLE;MALFERTHEINER, 2014; WISCHMEYER et al., 2017).

Para ser categorizado com probiótico, o microrganismo necessita preencher uma série de requisitos sendo eles: 1) efetivos e seguros, 2) não patogênicos, 3) resistentes contra a digestão por enzimas pancreáticas ou entéricas, ao ácido gástrico e suco biliar, 4) apresentar capacidade de persistir no trato gastrointestinal, 5) capazes de prevenir a aderência à mucosa intestinal do patógeno, 6) capazes de influenciar atividade metabólica local e 6) de evitar o estabelecimento e/ou a replicação de enteropatógenos (LEE;SALMINEN, 2009). Esses pré-requisitos têm sido empregados pela comunidade científica mundial como o perfil padrão-ouro e geralmente são utilizados na seleção desses microrganismos.

Os probióticos reduzem ou eliminam as populações dos patógenos pela secreção de ácidos orgânicos (acético e lático) e redução do pH (OGAWA et al., 2001; FAYOL-MESSAOUDI et al., 2005; FOSCHI et al., 2017), competição por nutrientes e receptores de células epiteliais (FORESTIER et al., 2001; NOWAK;MOTYL, 2017), produção de peróxido de hidrogênio (PRIDMORE et al., 2008), bacteriocinas (CORR et al., 2007; RIAZI et al., 2009; BAHRI;CHAOUCHE, 2016; BOYANOVA et al., 2017), biosurfactantes (GUDIÑA et al., 2010; FARIQ;SAEED, 2016; MORAIS et al., 2017).

### 2.2.1 Probióticos na terapêutica e prevenção das infecções intestinais bacterianas

Nos últimos anos a possibilidade das diversas aplicações dos probióticos tem representado uma estratégia interessante para o tratamento e a prevenção de algumas doenças infecciosas, principalmente das infecções intestinais, tais como a doença inflamatória intestinal (DII), síndrome do cólon irritável, diarreia infecciosa e associada ao uso de antibióticos (REID, 1999; TALESKI;TRPCEVA;ZDRAVKOVSKA, 2017; TALESKI et al., 2017).

Em um modelo de colite hemorrágica induzida pela infecção com *Escherichia coli* O157:H7 em camudongos, verificou-se que os isolados *B. longum* subsp. *longum* JCM 1217T, *B. longum* subsp. *infantis* JCM 1222T e *B. adolescentis* JCM 1275T protegeram o epitélio via

produção de acetato e inibição da translocação da toxina de Shiga do lúmem intestinal para o sangue (FUKUDA et al., 2011).

Existem evidências de que *Lactobacillus acidophilus* R0052 e *L. rhamnosus* R0011 reduzem a lesão sobre células intestinais em cultura de tecidos causadas por EPEC e EHEC, bem como inibem a adesão dessas bactérias às células T84 (SHERMAN et al., 2005). Ainda nesse contexto, foi observado que *L. acidophilus* Bar13 e *L. plantarum* Bar10, bem como *Bifidobacterium longum* Bar33 e *B. lactis* Bar30 podem realizar um deslocamento *in vitro* de ETEC e *Salmonella* em células de cólon HT-29 (CANDELA et al., 2008). Foi verificado também que o isolado *L. plantarum* CGMCC No.1258 pode atenuar as alterações no citoesqueleto provocadas por EIEC *in vitro* (QIN et al., 2009).

Um estudo que avaliou o efeito de *B. longum* HB25 modificado com o gene de um peptídeo antimicrobiano LL-37 secretado pelo epitélio intestinal, constatou que o microrganismo recombinante reduziu os níveis da citocina pró-inflamatória TNF-α e aumentou a dosagem da citocina anti-inflamatória IL-10 e o fator de crescimento transformante (TGF-α). Após a análise histológica, constatou-se também que houve uma redução das injúrias geradas à mucosa do cólon (GUO et al., 2017).

Um trabalho que analisou os efeitos de *Lactobacillus johnsonni* CJLJ103 em cultura de células e modelo animal, observou uma inibição do crescimento de *E.coli* e da produção de lipopolissacarídeo (LPS), além do aumento da expressão das proteínas das *tight junctions* em células Caco-2 e em camundongos com colite induzida pelo ácido 2,3,6-trinitrobenzenosulfonico (TNBS) (LIM et al., 2017).

Uma cepa de *Lactobacillus plantarum* designada de L9 inibiu a adesão e internalização de *E. coli* O55:B5 em monocamadas de enterócitos. Adicionalmente, foi observado que o microrganismo reduziu o fator de necrose tumoral (TNF-α) induzido pela translocação bacteriana em células Caco-2. Por último, foi constatado também que *L. plantarum* L9 não afetava a integridade das monocamadas celulares (WANG et al., 2017).

Além disso, através de sua intensa atividade fermentativa, algumas linhagens podem inibir a produção de toxinas e dessa forma atenuar a virulência de um enteropatógeno. Nesse contexto, a administração oral da cepa *Bifidobacterium breve* em murinos inibiu a produção da toxina Stx de EHEC O157:H7 pela elevada secreção de ácido acético e redução do pH intestinal (ASAHARA et al., 2004).

Portanto, considerando a importância da investigação de medidas alternativas para a atenuação da doença diarreica e que existem lacunas sobre a efetividade dos probióticos existentes sobre os principais enteropatógenos bacterianos, este estudo realizou uma caracterização de diferentes isolados de *Bifidobacterium* e *Lactobacillus* de fezes de recém-nascidos com perfil probiótico sobre diferentes linhagens de DEC, *Salmonella* e *Shigella*.

## 3 OBJETIVOS

GERAL

Realizar a prospecção de microrganismos com potencial probiótico em fezes de crianças contra enteropatógenos bacterianos de relevância clínica.

ESPECÍFICOS

- Selecionar isolados dos gêneros *Bifidobacterium* e *Lactobacillus* com as seguintes propriedades probióticas *in vitro*:
  - Tolerância à lisozima, pH e sais biliares;
  - Capacidade de adesão à mucina intestinal;
  - Secreção de metabólitos antimicrobianos;
- Avaliar a capacidade de adesão das cepas selecionadas à mucina gástrica;
- Investigar a capacidade de inibição da adesão de *Escherichia coli* diarreiogênicas, *Salmonella* Choleraesuis e *Shigella flexneri* em mucina por deslocamento;

## 4 RESULTADOS

4.1 CAPÍTULO 1 – Probiotics, mechanisms of action, and clinical perspectives for diarrhea management in children (publicado na revista Food & Function, Qualis B1 em Medicina I e Fator de Impacto 3.289).

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REVIEW



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# Probiotics, mechanisms of action, and clinical perspectives for diarrhea management in children

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Infectious diarrhea is the second most common cause of morbidity and mortality in children under 5 years of age in the underdeveloped areas of the world. Conventional treatment consists of rehydration, which may be coupled with antimicrobial agents in more severe bacterial infections or with antiprotozoal agents. In the last few decades, research on the use of probiotic strains, such as *Lactobacillus rhamnosus* GG ATCC 53013 (LGG), *Lactobacillus reuteri* DSM 17938 and *Saccharomyces boulardii*, has gained much attention to prevent and treat diarrheal diseases. However, they are rarely used in the clinical routine, perhaps because there are still gaps in the knowledge about the effective benefit to the patient in terms of the reduction of the duration of diarrhea and its prevention. Furthermore, only a few probiotic strains are safely indicated for usage in pediatric practice. This review summarizes the current knowledge on the antimicrobial mechanisms of probiotics on distinct enteropathogens and their role in stimulating host defense mechanisms against intestinal infections. In addition, we highlight the potential of probiotics for the treatment and prevention of diarrhea in children. We conclude that the use of probiotics is beneficial for both the treatment and prevention of diarrhea in children and that the identification of other candidate probiotics might represent an important advance to a greater reduction in hospital stays and to prevent infectious diarrhea in a larger portion of this population.

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## 1. Introduction

At a time marked by antimicrobial abuse and the serious problem posed by the emergence of multidrug-resistant bacteria, investing in alternative approaches for the prevention and/or treatment of infectious diseases is crucial. Gastrointestinal diseases can be caused by a wide variety of bacterial, viral and parasitic agents in both adults and

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children. Although many of the diarrheal cases are caused by viruses, an increased number of bacterial diarrhea have been reported, especially in areas with detrimental conditions of sanitation and hygiene.[1] Bacterial diarrhea accounts for 50–60% of the cases in developing countries.[2] Diarrhea is defined as three or more liquid or loose stools per day accompanied or not by fever and vomiting, being a self-limited condition.[3] The most serious cases have been reported in children, especially those under 5 years of age.[4] According to estimates by the United Nations Children's Fund (UNICEF), diarrhea is an important cause of morbidity and mortality in children under 5 years of age.[5] The most recent data indicate that approximately 480 000 children died due to diarrhea in this age group, representing 8.4% of child deaths worldwide in 2016.[6]

Of importance, children represent the predominant group who die of diarrhea in developing countries.[6] Cases in this group have been associated with diarrheagenic *Escherichia coli* strains,[7–9] *Salmonella* spp. and *Shigella* spp.[10] However, the data obtained by a recent global surveillance demonstrated that acute watery diarrhea in children under 5 years of age is mainly caused by rotavirus, especially in Africa, followed by norovirus GII, *Cryptosporidium*, *Shigella*/enteroinvasive (EIEC) and enterotoxigenic *E. coli* (ETEC), and enteric adenovirus 40/41.[11]

The main therapeutic measure for diarrhea applied in healthcare services, especially when viruses cause this condition, consists of oral or intravenous administration of saline solution (0.9%) or zinc (10–20 mg per day for 10 to 14 days), in order to prevent or correct dehydration.[12] However, this therapy is unable to eliminate all of the deleterious effects of infection (*i.e.*, duration of diarrhea and number of stools) or effectively restore the gut microbiota.[12,13] For this reason, several studies have investigated medications likely to have palliative or therapeutic actions against diarrhea, such as loperamide and diosmectite, whose major mechanism of action is reduction of intestinal motility; however, the reports about their benefits are not consistent.[14,15]

In patients with parasitic diarrhea (*e.g.*, amebiasis and giardiasis), adequate antiparasitic chemotherapy can shorten the clinical course of the disease and the duration of the elimination of pathogens in stools.[16] Empiric antibiotic regimen for routine acute bacterial diarrhea is discouraged, except for patients with severe bacterial (*e.g.*, shigellosis, cholera, and typhoid fever) infections. The first-choice treatment for more severe cases of diarrhea consists of the administration of antimicrobial agents. In these cases, fluoroquinolones (*e.g.* ciprofloxacin or levofloxacin) are generally indicated. In patients with diarrhea caused by *Campylobacter*, which can be fluoroquinolone resistant, the use of azithromycin for treatment can also be recommended.[17]

However, when broad-spectrum antibiotics, such as some penicillins (*e.g.* ampicillin, amoxicillin alone or with clavulanate, carbapenems and piperacillin/tazobactam), third- and fourth-generation cephalosporins, fluoroquinolones, tetracyclines, chloramphenicol, and so on, are selected for empiric treatment or when treatment fails due to resistance to the chosen drug, the spread of resistance to other bacteria might be facilitated.[16,18] Recently, a metagenomic study on gut microbiome genes from the sequencing data of 162 people (85 Danish, 39 Spanish and 38 Chinese individuals) has revealed that the human gut microbiota can function as a reservoir for several antibiotic resistance genes, principally tetracyclines, macrolides, and beta-lactams.[19] So, indiscriminate antibiotic use can certainly exert a selective pressure on the gut microbiota and thus contribute to the current emergence of resistance worldwide.

Within this context, several studies have investigated the use of probiotic microorganisms as alternative therapies to attenuate infection. Probiotics are living microorganisms that, when administered in adequate amounts, can result in several benefits for the host, such as microbiota recovery, improve-

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ment of the immune system and food absorption.[20] Probiotics can be delivered lyophilized in capsules and sachets or can be added to foods, such as yogurt or other dairy products.[21–23]

In recent years, a rise in the amount of probiotic consumption has been observed, especially of dairy products. This is due to the increased knowledge of their mechanisms of action and the benefits they may present, which have been vastly advertised to the global community. The probiotic market has been projected to exhibit a progressive 7.4% compound annual growth rate between 2014 and 2020. In 2014, according to a Transparency Market Research report, its global market was valued at more than US$ 62 billion and is expected to reach US$ 96 billion by 2020.[24] The largest population of probiotic consumers is from the Asia-Pacific region, mainly China, Japan and India. In Europe, the United Kingdom and Germany represent the most profitable market. In this scenario, dairy products are the largest market for the application of probiotics and are estimated to achieve a $ 32.2 billion profit by 2018.[25]

The beneficial effects of fermented milk beverages have been investigated in diarrhea; however, their efficacy to either prevent or treat this condition is still a matter of debate. Indeed, their effects have been assessed in individuals of different ages with different diarrheic conditions. Probiotic-enriched milks, yogurt and kefir have been assessed in trials as preventive approaches in diarrhea and most of the studies showed promising potential for these dairy products in reducing diarrhea duration and number of episodes. A placebo-controlled randomized trial with 369 patients showed that the administration of yogurt at the time of antibiotic therapy is not effective in reducing the antibiotic-associated diarrhea (AAD) incidence. Of these patients, 131 received Bio yogurt, which contained *Streptococcus thermophilus*, *Lactobacillus acidophilus*, and *Bifidobacterium animalis* ssp. *lactis* (total daily dose of $10^9$ bacteria in 150 mL for 12 days), while 118 received commercial yogurt and 120 did not receive intervention with yogurt.[26] Similar outcome was found in patients receiving 150 mL of kefir for 10 days.[27] In contrast, a previous study by Beniwal *et al.*, comprising 202 individuals, found a 50% reduction in the occurrence of AAD and a reduction in the duration of diarrhea (23 *versus* 60 days) in those taking a probiotic yogurt containing a mixture of *L. acidophilus*, *Lactobacillus bulgaricus* and *S. thermophilus* ($10^6$ cultures per g; twice daily, for 8 days).[28]

As noted for AAD, probiotic-enriched milk drinks have also been tested in acute and persistent diarrhea. A study by Chouraqui *et al.* demonstrated that the administration of a milk formula containing *Bifidobacterium lactis* strain Bb 12 (at least $10^8$ bacteria, indicated as colony-forming units or CFU) shortened the episodes of diarrhea and the number of days with diarrhea in infants for the duration of their stay in residential child care centers (range: 119.5–154.7 days).[29] Similarly, the administration of fortified milk containing *B. lactis* HN019 ($1.9 \times 10^7$ CFU) and prebiotic oligosaccharide three times a day for one year reduced dysentery episodes in children.[30] Similarly, the administration of 100 ml of a probiotic yogurt containing *L. bulgaricus*, *L. acidophilus*, *Bifidobacterium*, and *S. thermophilus* ($10^9$ CFU), for 5 days, reduced acute diarrhea episodes in children.[31]

It is important to highlight that many marketed products range from 1 to $10 \times 10^9$ viable cells per dose; however, whilst some products have proven to be effective at lower levels ($10^7$ CFU per dose), others have required more probiotic colonies to achieve similar effects ($10^{10}$ CFU per dose).[32–35]

All these pieces of evidence of variability suggest that the effective dose required for achieving beneficial effects on diarrhea when taking probiotics vary with the strain(s) and the formulation of the probiotic product. Therefore, larger clinical trials are needed in order to define the best probiotic options and the ideal duration of treatment for diarrhea.

In addition to the effects on infectious diarrhea, other beneficial effects of probiotic intake have been reported. These include improvement of lactose intolerance (the yogurt bacteria release lactase when lysed by bile acids),[36] reduction of

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allergic reactions of milk-fed infants to casein (some lactobacilli degrade this protein into smaller peptides and amino acids),[37] reduction of serum cholesterol levels and blood pressure,[38,39] and relief of constipation.[40] As a result, the consumption of probiotics has increased in recent years.

Here, we reviewed the use of probiotics in the treatment and prevention of pediatric diarrhea. We particularly focused on their mechanisms of action on distinct etiological agents, efficacy, and safety and we discussed the *pros* and *cons* related to the use of probiotics for diarrhea in children.

## 2. Mechanisms of action

### 2.1. Modification of host defenses

The main mechanisms of action of probiotics against enteropathogenic agents are depicted in Fig. 1. During intestinal bacterial infection, there is colonization of the gut epithelium by the pathogen. The mucus produced by goblet cells accounts as one of the first lines of defense against pathogenic bacteria colonization[41] and the intestinal cells produce antimicrobial peptides (cathelicidins and defensins) in order to contain the infection.[42]

Cathelicidins are small, cationic, antimicrobial peptides found in vertebrates that have a broad range of action against bacteria, fungi, protozoa, and some viruses. They are stored inside secretory granules of neutrophils and macrophages and are released after leukocyte activation. Expression of cathelicidins can also occur in non-myeloid cells, such as epithelial cells of the testis, skin, and the gastrointestinal and respiratory tracts.[43]

Defensins are short, cationic, cysteine-rich polypeptides that are important in innate immunity against a variety of pathogens. They are involved in adaptive immunity, including relevant roles in inflammation, wound repair, expression of cytokines and chemokines, production of histamine and enhancement of antibody responses.[44]

The intense interaction of the pathogen and the gut promotes the capture of the pathogenic bacteria by dendritic cells, which in turn process their antigens and present them to T cells. Activated T cells release pro-inflammatory cytokines as part of the host's response to infection,[45] resulting also in the activation of antibody-producing B cells, particularly delivering IgA specific to the infectious pathogen (Fig. 1A).

It has been demonstrated that the administration of probiotics modifies these responses. The modifications are summarized in Fig. 1B and are discussed herein. Indeed, probiotics stimulate intestinal goblet cells to produce mucins, a family of glycoproteins composing the gut mucus.[46] Among the 18 different types of polymeric glycoproteins that comprise the mucin family in humans, MUC2 (mucin 2) is the main component of the intestinal mucus. As mentioned above, mucus production is crucial for the protection of the host against pathogen colonization and invasion and is essential for the lubrication of the intestinal epithelium.[47] *In vitro* evidence indicates that several *Lactobacillus* species increase mucin secretion by various intestinal epithelial cell lines, such as the Caco-2 (MUC2), HT-29 (MUC2 and MUC3) and LS174 T (CmbA and MUB) cell lines, with consequent blocking of the adhesion and invasion of pathogenic *E. coli* strains and other pathogens.[46,48,49] Probiotics also enhance the production of cathelicidins and defensins by the gut epithelium.[50,51]

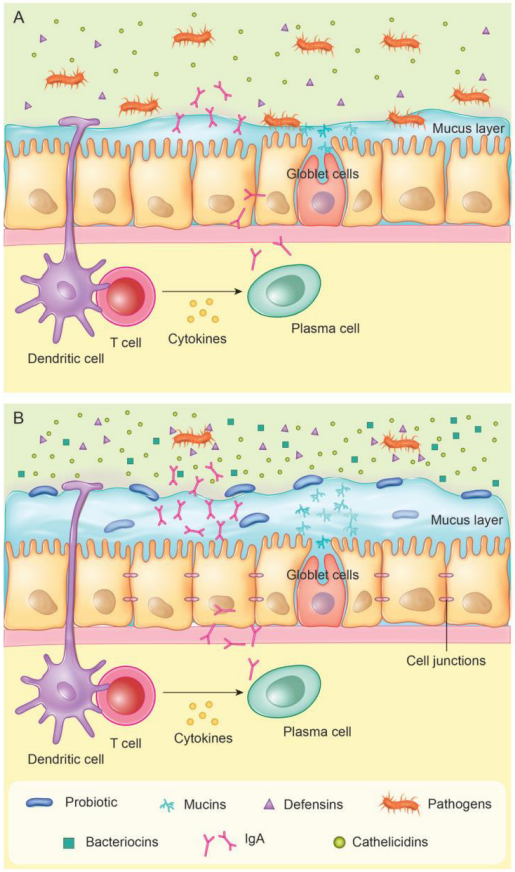


**Fig. 1** Mechanisms of probiotic action against enteropathogenic microorganisms. (A) Immune response during intestinal bacterial infection in the absence of probiotics: (i) there is colonization of the gut epithelium by the pathogen, (ii) mucin production by goblet cells, (iii) production of antimicrobial peptides (defensins and cathelicidins) by intestinal cells, (iv) phagocytosis of pathogenic bacteria and antigen presentation to T lymphocytes by the dendritic cells, (v) cytokine release by activated T cells, and (vi) activation of B cells and subsequent release of antibodies, particularly IgA, which are specific to the infectious pathogen. (B) Protective mechanisms of probiotics against intestinal bacterial infection: (i) stimulation of mucin production by intestinal goblet cells, (ii) induction of the production of antimicrobial peptides, (iii) improvement of cell junction stability, (iv) increase in IgA release by activated B cells, and (v) inhibition of pathogen growth and/or elimination of pathogen through the production of antimicrobial molecules such as short-chain fatty acids, bacteriocins and microcins.

### 2.2. Effects on the cytoskeleton and tight junctions

Different enteropathogens cause the rupture of junctional proteins and actin contraction in enterocytes, resulting in increased permeability in the intestinal lumen.[52–55] It has been suggested that probiotics prevent tight junction disruptions in these cells following infectious and/or inflammatory insults.[56–60] A similar effect for probiotics was observed in enterocytes with tight junctions that had already been disrupted by EHEC.[58]

Indeed, the stability of cell junctions induced by probiotics is due to the increased expression of proteins composing tight junction strands, such as claudin-1, occludin, zonula occludens 1 (ZO-1) and junctional adhesion molecule 1 (JAM-1).[61–64] The latter reduces the entrance of pathogens and their metabolites into the intestinal epithelium. Studies with a mixture of probiotic *Lactobacillus* strains have shown that these microorganisms are able to attenuate the damage induced by *Salmonella typhimurium* lipopolysaccharide (LPS) to cell junctions.[65] *S. thermophilus* ATCC19258 and *L. acidophilus* ATCC4356 conferred protection to the intestinal epithelium against EIEC by increasing transepithelial resistance, and maintaining or enhancing cytoskeletal and tight junctional protein phosphorylation.[66] Another study demonstrated that the *B. lactis* 420 cell-free supernatant increases the integrity of the tight junctions of intestinal epithelial cells and protects them against enterohemorrhagic *E. coli* (EHEC) O157:H7-derived products.[67] Secreted proteins from LGG, such as p40 and p75, were also shown to be able to protect the intestinal epithelial tight junctions against hydrogen peroxide-induced damage *via* protein kinase type C (PKC)- and mitogen activated protein (MAP) kinase-dependent mechanisms.[68] Furthermore, the pretreatment of Caco-2 monolayers with *Lactobacillus plantarum* attenuated phorbol ester-induced dislocation of ZO-1 and occludin from the intercellular junctions and the increased epithelial permeability.[61]

In this context, these pieces of evidence suggest that children with diarrhea may benefit from both preventive and therapeutic approaches with probiotics.

### 2.3. Induction of IgA production

IgA production by activated B cells is an important process that contributes to the resistance of the intestinal mucosa against infections. Mounting evidence showed that probiotics increase IgA levels in the mucosa and luminal mucus layer,[69,70] whilst its soluble form can be detected in stool samples following their repeated ingestion.[71] IgA provides the immune exclusion of pathogens by binding to their antigens, thus, forming a hydrophilic shell on their surface, which is repelled by the mucosal glycocalyx.[72,73] Antigen–IgA complexes also bind to the receptor FcRI constitutively expressed on innate immune cells,[73,74] triggering their activation, and in turn, antimicrobial and inflammatory responses.[73,75] Of note, these processes protect the epithelium not only against pathogen invasion but also against excessive growth of enteric bacteria, which might occasionally become opportunistic.[76,77]

Probiotics are suggested to enhance IgA release in the gut by directly acting on the mucosal immune system by various mechanisms. For example, genomic DNA from LGG and *B. animalis* subsp. *lactis* Bb12 was found to increase Toll-like receptor 9 (TLR9) expression in mucosal mononuclear cells and enhance the maturation of dendritic cells in the intestine.[78,79] Probiotic genomic DNA of *Bifidobacterium longum* and LGG may also activate B lymphocytes.[80,81] Additionally, IgA release may be the result of the activation of T cells by probiotics.[78] Irrespective of T cell activation, the IgA production triggered by these microorganisms can be mediated by enhanced release of cytokines such as transforming growth factor β (TGF-β), interleukin-6 (IL-6) and IL-10.[78,82] TGF-β expression in dendritic cells was up-regulated by not only *Bifidobacterium* sp. but also by lactobacilli strains such as *Lactobacillus gasseri* SBT2055, an effect shown to contribute to IgA production in the small intestine *via* TLR2 activation.[82]

### 2.4. Antimicrobial activity by short-chain fatty acids

Probiotics themselves are able to produce short-chain fatty acids by fermentation.[83,84] Indeed, probiotics promote the reduction of the intestinal lumen pH through the production of organic acids principally during the metabolism of carbohydrates, with consequent inhibition of pathogen growth.[85] The major organic acids produced by species of *Bifidobacterium*, *Clostridium*, *Streptococcus*, and *Lactobacillus* include: acetic, butyric, propionic and lactic acids. Due to its dissociation constant (p*K* value), acetic acid (p*K* value of 4.756) is considered to have a higher antimicrobial activity than lactic acid (p*K* value of 3.860).[83]

In their undissociated forms, short-chain fatty acids are antimicrobial against both Gram-negative and Gram-positive bacteria; this is due to their lipophilicity and ability to solubilise in bacterial cell membranes.[83,86,87] Once in the bacterial cytoplasm, these acids ionize to yield protons, thus, interfering with the permeability of the pathogen cell membrane, promoting uncoupling of both the substrate transport and oxidative phosphorylation from the electron transport system.[83,86,87] Also, these short-chain fatty acids can cause structural damage to the cell membranes of Gram-negative bacteria increasing their permeability to other small antimicrobial molecules, such as hydrogen peroxide and reuterin.[88,89]

### 2.5. Role of bacteriocins and microcins

Bacteriocins and microcins are small peptides with bactericidal or bacteriostatic activity produced by both probiotics and commensal bacteria. The term bacteriocin is used to designate peptides secreted by Gram-positive bacteria, while microcin is used to designate peptides produced by Gram-negative bacteria, being thus named for having sizes smaller than 10 kDa.[90] Bacteriocins are suggested to increase the permeability of the inner membrane of Gram-negative bacteria, thus contributing to its rupture, and to interfere with bacterial cell wall synthesis, resulting in pore formation by binding to the peptidoglycan precursor lipid II.[91] Indeed, the bacteriocin lacticin 3147, produced by *Lactococcus lactis*, forms selective

 

pores in the cell walls of some pathogenic Gram-positive bacteria, including *Clostridium difficile*, resulting in their killing.[92,93] Recently, the bacteriocin ABP-118, produced by *Lactobacillus salivarius*, has been shown to inhibit the *in vitro* growth of *Bacillus*, *Listeria*, *Enterococcus* and *Staphylococcus.*[94] In contrast, microcins act directly on the inner membrane or inhibit the activity of enzymes involved in the structure and synthesis of DNA or RNA, with consequent destruction of the pathogen.[95] Microcin J25 is overproduced by an *E. coli* strain and shows inhibitory activities against EHEC (O157:H7).[96]

### 2.6. Competition for adherence sites

Importantly, probiotics can also hinder or fully prevent pathogen colonization by competing for binding sites in mucin or epithelial cells. A study conducted with *Lactobacillus reuteri*, *L. gasseri* and *L. plantarum* showed that these species inhibit EIEC and EHEC adhesion to and invasion of HT-29 cells seemingly *via* blocking of cell receptor binding sites.[97] Moreover, the Ip_1643 protein expressed by *L. plantarum* WCFSI was found to inhibit the adhesion of *Listeria monocytogenes*, *S. typhimurium* ATCC14028 and *Shigella flexneri* CMCC(B) 51572 to gastric mucin by more than 95%.[98]

Several assays have been used to investigate the ability of a probiotic to colonize the intestinal mucosa. Cell lines derived from human colon adenocarcinomas such as Caco-2 and HT-29 are generally employed.[99–102] Alternatively, mucin obtained from the human colon[103–105] or animals[106] has been employed. Both methods can be used for the *in vitro* prediction of probiotic potential to colonize the gut; however, some *ex vivo* assays with human or animal intestinal mucosal tissue have also been employed.[107,108] Although this is a good method for the evaluation of probiotic adhesion to the gastrointestinal epithelium and mucus, it is not feasible to be routinely performed due to the difficulties of obtaining intestinal mucosa samples. Alternatively, the culture of stool samples obtained from individuals taking probiotics could be used to monitor *in vivo* intestinal colonization.[109]

### 2.7. Mechanisms of action against the main enteric viruses

The use of probiotics has been associated with beneficial effects for the prevention and treatment of rotavirus diarrhea.[110] The mechanisms that account for these effects against viral pathogens have not been fully elucidated. It has been reported that probiotics interfere with the viral cycle by specific and non-specific mechanisms.[111,112] Also, *Bifidobacterium breve* DSM20091, *B. longum* Q46, *Lactobacillus paracasei* A14, *L. paracasei paracasei* F19, *L. paracasei*/*rhamnosus* Q85, *L. plantarum* M1.1, and *L. reuteri* DSM12246 can inhibit the adsorption of the virus to the intestinal cells.[113] This mechanism may involve steric hindrance, or improvement of the barrier effects of the mucus, glycocalyx, and intercellular junctions.[66,67,114]

Additionally, probiotics can inhibit virus adsorption by competing with viral receptors on intestinal cells.[115] Recently, Fernandez-Duarte *et al*. have suggested that protein-based metabolites from *Lactobacillus casei* Lafti L26-DSL and *Bifidobacterium adolescentis* DSM20083 can block rotavirus entrance by a direct effect on the virus particle.[116]

Increases in the humoral response and greater proliferation of IgA-, IgM- and IgG-secreting B cells have been reported to occur during the acute phase of viral diarrhea in children treated with LGG. In fact, approximately 90% of the participants in the study receiving probiotics developed specific IgA responses against rotavirus by the convalescence stage.[117]

Based on the findings of a mouse model of protection with *Bifidobacterium bifidum* G9-1 against rotavirus infection, it was proposed that oral administration of this probiotic strain can promote both prophylactic and therapeutic effect for rotavirus diarrhea by the induction of mucosal protective factors.[118] After probiotic use, the number of mucin-producing cells in the small intestine was increased as well as the gene expression levels of MUC2, MUC3, MUC4, TGF-β and TFF3, which are related to mucosal protection.

In addition to the effects against rotavirus infection, probiotic strains also have the potential to prevent and treat norovirus infection. Studies with a mixture of LGG and *E. coli* strain Nissle 1917 resulted in reduced incidence and diarrhea duration in a gnotobiotic pig model.[119] The beneficial effects were associated with stimulation of interferon-γ (IFN-γ) T cell responses, enhanced intestinal immunity and promotion of intestinal epithelium health and growth.

Taken together, these data suggest that probiotic strains can display multiple mechanisms for the amelioration of enteric virus infection that can act directly on the microorganism and by the modulation of intestinal homeostasis.

### 2.8. Mechanisms of action against the main intestinal protozoa

Few studies have described the potential actions of probiotics against major intestinal protozoan parasites, such as *Entamoeba*, *Giardia* and *Cryptosporidium*.[120,121] With respect to amebiasis, a decrease in bloody diarrhea caused by *Entamoeba histolytica* was observed in a clinical trial when *Saccharomyces boulardii* was administered together with metronidazole to children.[122] The putative mechanisms of action are not entirely known, but it was proposed that *S. boulardii* can inhibit the binding of *E. histolytica* to human cells, since amoeba cells interact with *N*-acetylgalactosamine residues on yeast cells.[123] Recently, it has been shown that a combination of *L. casei* NCDC299 and *Enterococcus faecium* NCDC124 can reduce the viability and infectivity of *Entamoeba in vitro*.[120]

On the other hand, *Lactobacillus johnsonii* LA1 produces a heat-sensitive low molecular weight compound with the ability to inhibit *Giardia lamblia* growth *in vitro*, without interfering with its adhesion to epithelial cells.[124] Oral administration of the *L. johnsonii* LA1 strain protected gerbils against *G. lamblia* infection apparently by stimulating the immunity of the animals against the parasite.[125] In addition to enhancing host immune response, probiotics also modulate *Giardia* infection by interfering with parasite adhesion to intestinal mucosa, as has been described for the *L. casei* MTCC1423 strain.[126]

It is premature to say that probiotics can be an alternative strategy to treat patients with cryptosporidiosis, since only a single case report of successful treatment in humans has been reported. A 12-year-old girl took LGG and *L. casei* Shirota for 4 weeks and within 10 days of treatment, the diarrhea and nausea had ceased.[127] Apparently, the mechanism of action is not associated with host immune stimulation, but instead with a probiotic compound that reduces oocyst shedding of *Cryptosporidium* spp. as observed in an animal model using *L. reuteri* (strains 23272, 4000 and 4020) or *L. acidophilus* NCFM.[121]

## 3. Use of probiotics for the treatment of distinct types of diarrhea in children

Until a few years ago, probiotics were considered a form of alternative medicine; however, advances in discoveries made during their actual use have provided more consistent evidence of the benefits associated with these agents, which could thus be included as a relevant component of routine medical care. A series of studies assessed the use of probiotic microorganisms for diarrhea treatment in children (Table 1). However, only a few species that are well-documented species have been widely used in clinical practice, despite some controversies.[128] In many clinical situations probiotics can be recommended to treat diarrhea in children, but the major indications are described below.

### 3.1. Acute diarrhea

One of the most recommended probiotic strains for bacterial, parasitic and viral diarrhea treatment in children is LGG.[129–131] The beneficial effects in treatment have also been reported with other probiotic strains, such as *L. reuteri*,[132–135] *L. acidophilus*,[136] *B. lactis*,[137] and *Saccharomyces boulardii*.[138,139]

Regarding viral diarrhea, it was shown that early intervention with LGG ($10^9$ CFU) in a group of children reduces the number of stools and the duration of diarrhea from 76.6 ± 41.6 h (placebo group) to 56.2 ± 16.9 h.[130] A meta-analysis found that a dose $\geq 10^{10}$ CFU of LGG once a day sufficed to effectively reduce the length of hospital stays and the duration of viral diarrhea by 25.2 h.[140] It is important to highlight that this dose was more efficacious when used in European children than in those from developing or underdeveloped countries, as in the non-European children the difference in the duration of diarrhea between the LGG group and the control group had only borderline statistical significance (six randomized clinical trials, $N = 1700$; mean difference −0.87; 95% CI −1.81–0.08). An explanation for such results can be attributed to differences in diet. In fact, food or diet substrates are considered important factors in the regulation of the gut microbiota. Foods may help in partially neutralizing the acidic pH of the stomach or contain other functional ingredients that stimulate the functionalities of probiotics. Food components that induce the growth of probiotics are called prebiotics;[141] for instance oligosaccharides such as lactulose, galactooligosaccharides, inulin, fructooligosaccharides, and other dietary carbohydrates or fibers not digestible by humans are used by probiotics.[142] Thus, the consumption of healthier foods has become a large trend, which has quickly gained space in the last few decades in European countries and is now becoming a hit amongst other populations. Still, dietary patterns are suggested to be population specific and influenced by sociocultural factors and food availability;[143,144] thus, these factors may influence the chances of success of probiotic-based treatments, as the amount of daily consumption of prebiotics and even probiotic-containing products may have previously shaped or not the intestinal microbiota towards a beneficial pattern. Importantly, food consumption may not be the only predisposing factor for probiotic therapy success as many others such as medications, physical activity, cultural habits, immunity status and genetics, amongst others, may also alter the gut microbiota.[145]

It is also possible that the limited effect of LGG in non-European children is due to the higher frequency and diversity of enteric pathogens with different virulence mechanisms in this population (*Campylobacter*, *Salmonella*, *Shigella*, *Yersinia* and *Entamoeba*), which may hinder the action of probiotics. The presence of these microorganisms is directly related to the precarious hygiene and sanitary conditions in those countries. Of importance, diarrhea is usually caused by viruses in Europe.[1] Interestingly, LGG is more efficacious among outpatients than among inpatients. Patients who require a long stay at the hospital are subject to secondary infections, acquisition of infections caused by resistant bacteria, and diarrhea that may be worsened by inadequate or prolonged antibiotic treatment. These factors may explain the differences in the range of action of probiotics.[140]

On the other hand, controversial data on the efficacy of probiotics have also been reported. The administration of *L. paracasei* strain ST11 to children aged 4 to 24 months with viral diarrhea induced neither daily nor cumulative reduction of the number of stools (during the six days evaluated) in comparison with the placebo group.[146] Indeed, the authors observed that in comparison with other studies, diarrhea lasted longer (4 *versus* 1.6–3.8 days) and the number of stools was higher (10 *versus* 2.5–7) in children who received this probiotic.

It is worth noting that the efficacy of probiotics is related to several factors, including dose and ability of intestinal colonization. In children aged 6 months to 3 years, watery diarrhea caused by rotavirus persisted only in 26% of the patients who received $10^{10}$–$10^{11}$ CFU of *L. reuteri versus* 81% in the group administered placebo on the second day of follow-up. This difference was due to the efficacious colonization of probiotics in the gut and the rapid expression of *L. reuteri* properties as early as 48 hours after the onset of treatment. At this time point, the CFUs of *L. reuteri* in the treated group had reached a 5-$\log_{10}$ increase.[147]

The probiotic actions of *L. reuteri* may be also related to the improved elimination of pathogenic bacteria, which are nor-

 

**Table 1** Use of probiotics for the treatment of diarrhea in children

| Administered probiotic or formula | Dose | Study design | Sample size (treatment group/ placebo group) | Age range | Type of diarrhea (etiologic agent) | Outcomes | Study |
|---|---|---|---|---|---|---|---|
| *L. acidophilus* (80%), *L. bulgaricus* (10%), *B. bifidum* (5%) and *S. thermophilus* (5%) | $10^9$ CFU | Inpatients | 290 (65/225) | 1 year and 9 months–2 years | Bacterial and viral | No effects on diarrhea and associated symptoms | 169 |
| *B. lactis* | $14.5 \times 10^6$ CFU per 100 ml of formula of milk for 7 days | Inpatients | 50 (25/25) | <2 years | Essentially viral | Reduction in the duration of diarrhea (24 h) and hospitalization time (48 h) | 137 |
| *B. longum*, *B. lactis*, *L. acidophilus*, *L. rhamnosus*, *L. plantarum* and *P. pentosaceus* | $10^9$ CFU | Outpatients | 29 (6/23) | 3 months–7 years | Viral (rotavirus) | Reduction in the duration of diarrhea (~2 days) and vomiting (1 day) | 111 |
| *E. coli* Nissle | $10^8$ CFU | Outpatients | 151 (75/76) | 1 months–4 years | Bacterial and viral | Effective in treating diarrhea lasting longer than 4 days | 212 |
| LGG | $10^9$ CFU | Inpatients | 179 (90/89) | 3 months–3 years | Bacterial and viral | No effects on diarrhea and associated symptoms | 131 |
| LGG | $10^{10}$ CFU per 250 mL of ORS | Inpatients | 287 (147/140) | 1 month–3 years | Viral (rotavirus) | Reduction in the duration of diarrhea (~22 h) and hospitalization time | 130 |
| | $10^9$ CFU | Outpatients | 200 (100/100) | 6 months–5 years | Viral (rotavirus) | Reduction in the duration of diarrhea (~18 h) and improvement of stool consistency | 129 |
| LGG; *S. boulardii*; *B. clausii*; *L. delbrueckii* var *bulgaricus*, *S. thermophilus*, *L. acidophilus*, *B. bifidum* and *E. faecium* SF68. | $10^7$–$10^9$ CFU | Outpatients | 571 | 3 months–3 years | Viral | Reduction in the duration of diarrhea by LGG (37 h) or the mixture (45 h), and in the number of bowel movements | 170 |
| *L. paracasei* ST 11 | $5 \times 10^9$ CFU, 2× per day for 5 days | Inpatients | 230 (115/115) | 4 months–2 years | Bacterial and viral | Reduction in the cumulative stool output, stool frequency (in 14.6) and ORS intake | 146 |
| *L. reuteri* DSM 17938 | $1 \times 10^8$ CFU + ORS for 5 days | Outpatients | 60 (29/31) | 3 months–5 years | Viral | Reduction in the duration of diarrhea (15 h) | 132 |
| | $10^8$ CFU | Inpatients | 127 (64/63) | 3 months–5 years | Viral | Reduction in the duration of diarrhea (35 h) and hospitalization time (24 h) | 133 |
| | $4 \times 10^8$ CFU | Inpatients | 69 (35/34) | 6 months–3 years | Viral | Reduction in the frequency and duration of diarrhea (1.2 days) | 134 |
| | $10^8$ CFU $day^{-1}$ | Inpatients | 60 (30/30) | 3 months | Bacterial | Reduction in the colonization of enteropathogens | 135 |
| *L. rhamnosus* 19070–2 and *L. reuteri* DSM 12246 | $10^{10}$ CFU | Outpatients | 43 (24/19) | 9 months–4 years | Bacterial and viral | Reduction in the duration of diarrhea (40 h) | 213 |
| LGG | $6 \times 10^7$ CFU | Inpatients | 684 (330/332) | >1 year | Viral (rotavirus) | No effects on diarrhea and associated symptoms | 214 |
| *L. sporogenes* | $6 \times 10^7$ CFU | Inpatients | 148 (78/70) | 6–24 months | Viral (rotavirus) | No effects on diarrhea and associated symptoms | 215 |
| *S. boulardii* | 250 mg $day^{-1}$ | Inpatients | 200 (100/100) | 3 months–7 years | Bacterial, parasitic and viral | Reduction in the duration of diarrhea (~24 h) and hospitalization time (24 h) | 216 |
| | 500 mg $day^{-1}$ for 5 days | Inpatients | 60 (30/30) | 3 months–5 years | Viral (rotavirus) | Reduction in the duration of diarrhea (29 h) and hospitalization time (17 h) | 138 |
| | 250 mg, 1× or 2× for six days (depending on the age) | Outpatients | 88 (44/44) | 3 months–2 years | Viral | Reduction in the duration of diarrhea (~1.5 days) and frequency of stool elimination | 139 |
| *S. boulardii* + metronidazol | 250 mg, 2× per day for 7 days | Inpatients | 50 (25/25) | 10–12 years | Parasitic (*Entamoeba histolytica*) | Reduction in the duration of bloody diarrhea (30 h) and improvement of the elimination of parasite cysts | 122 |

CFU: colony forming units. LGG: *L. rhamnosus* GG. ORS: oral rehydration solution.

mally producers of urease.[148] Patients taking this probiotic exhibit lower urease levels than those in the control group. This is suggested to be related to the ability of *L. reuteri* to produce a powerful antimicrobial metabolite – a glycerol-derived β-hydroxypropionaldehyde known as reuterin, which stimulates the host's immune response and stabilizes the mucous membrane through the reduction of the intestinal permeability.[149] When used at a similar dose ($1 \times 10^8$ CFU), *L. reuteri* DSM 17938 was shown to reduce diarrhea in outpatient and hospitalized children in approximately 15 and 35 h, respectively.[132,133]

Another probiotic suggested to be effective in children is *B. lactis*. A randomized, double-blind study conducted in Egypt found that administration of $14.5 \times 10^6$ CFU of *B. lactis* to children aged 1–23 months for one week reduced the duration of diarrhea from 4.10 ± 0.94 to 3.12 ± 0.92 days. Furthermore, the number of stools per day decreased from 4.46 ± 0.85 to 3.96 ± 0.62 and the length of stay at the hospital was 28% shorter in the group given the probiotic.[137] In addition to its beneficial effects at a low concentration (compared to all other probiotics), the genus *Bifidobacterium* is known for exhibiting different mechanisms of resistance to bile salts which allow these bacteria to overcome the adverse conditions found in the gastrointestinal tract.[150]

While most of the microorganisms suggested as probiotics are bacteria, it is important to note that yeasts represent slightly less than 0.1% of the gut microbiota and that some species (*S. boulardii* and *Saccharomyces cerevisiae*) are used for large-scale treatment of diarrhea in children.[122] Indeed, it was shown that *S. boulardii* capsules reduce the duration of diarrhea from 6.16 to 4.70 days in children aged 2 months to 4 years in comparison with the placebo group. Apparently, timing is critical for treatment, since this probiotic was more effective when administered within the first 48 h following the onset of diarrhea, as colonization by *S. boulardii* during the first days of the disease is necessary for competition and consequent inhibition of pathogen adhesion.[139] In India, the duration of viral diarrhea in children with an average age of 2 years decreased from 89 to 60 h (95% CI: −41.2 to −16.8) following the administration of two 250 mg doses of *S. boulardii* within 5 days. In addition, the length of stay at the hospital decreased from 91 to 74 hours (95% CI: −33.46 to −0.54). No differences were found in the durations of either fever or vomiting.[138]

It is important to highlight that in the majority of the studies, probiotics were taken for 5–7 days after the onset of diarrhea. Their therapeutic effects included the reduction in the duration of diarrhea and the length of stay at the hospital, both within the first two days of treatment. It is possible that the timing of treatment interferes with probiotic efficacy in attenuating diarrhea and so, its prompt introduction at the onset of diarrhea may increase the chance of treatment success.

### 3.2. Persistent diarrhea

Probiotic-based therapy is a promising strategy as an adjuvant therapy in the treatment of children with persistent diarrhea caused by enteroaggregative *E. coli* (EAEC), which is associated with long-term gut inflammation. Inflammation may remain present even when the diarrhea is absent, but it may impair physical and cognitive development in children.[151,152] Probiotic intake in these cases would help to restore the gut microbiota and contribute to attenuate inflammation in the gut, since some microorganisms considered as normal members of the gut microbiota, such as *Faecalibacterium prausnitzii*, can display anti-inflammatory activities.[153] It is also possible to use a genetically modified probiotic strain that is able to express the anti-inflammatory cytokine IL-10 that was successfully used to treat colitis in an animal model.[154]

## 4. Use of probiotics for diarrhea prevention in children

In addition to the therapeutic use of probiotics in infectious diarrhea and other intestinal disorders, they also have potential for preventive use (Table 2). If administered within the first months of life, when the gut microbiota is being established, probiotic microorganisms may reduce the occurrence of other gastrointestinal diseases, such as necrotizing enterocolitis and sepsis with intestinal origin.

### 4.1 Acute diarrhea

The administration of *L. reuteri* DSM 17938 ($10^8$ CFU $day^{-1}$, once a day, for 30 days, 30 minutes before the first daily feed) to children with a mean age of 3 months contributed to maintain the balance of the gut microbiota by reducing colonization by opportunistic or pathogenic microorganisms, including Gram-negative anaerobic bacteria, enterococci, and enterobacteria. While the rate of infection with atypical enteropathogenic *E. coli* (a-EPEC) was 13.3% among the controls, no cases of infection occurred among the children previously colonized with the probiotic. Furthermore, increased populations of anaerobic Gram-positive bacteria were detected, which suggests greater proportions of the *Bifidobacterium* and *Lactobacillus* genera.[135] On the other hand, in a recent study, a higher daily dose of *L. reuteri* DSM 17938 ($1 \times 10^9$ CFU) in children 1–48 months of age was not effective in preventing nosocomial diarrhea.[155]

Malnourished Peruvian children (6–24 months old) treated with LGG ($3.7 \times 10^{10}$ CFU, once a day, 6 days per week, for 15 months) presented with fewer episodes of bacterial, parasitic and viral diarrhea (5.21 episodes of diarrhea per child per year *versus* 6.02 episodes in the placebo group; $p = 0.028$), which were more pronounced among 18–29 month old children and largely limited to non-breast-fed children, indicating that LGG supplementation decreases diarrhea incidence in high-risk children.[156]

Additionally, a mixture of LGG ($6 \times 10^9$ CFU) and micronutrients was shown to decrease the duration of hospitalization due to diarrhea (3.9 *versus* 4.9 days). It is worth noting that this study used a lower dose of LGG (1 log less) for a shorter

 

Table 2 Use of probiotics for the prevention of diarrhea in children

| Administered probiotic or formula | Dose | Study design | Sample size (treatment group/placebo group) | Age range | Type of diarrhea (etiologic agent) | Outcomes | Study |
|---|---|---|---|---|---|---|---|
| *B. animalis* subsp. *lactis* | $10^9$ CFU | Inpatients | 727 (362/365) | 1–18 years | Without previous diarrhea | No effects to prevent nosocomial infections | 217 |
| *B. bifidum* and *S. thermophilus* | *B. bifidum* ($1.9 \times 10^8$ CFU) + *S. thermophilus* ($0.14 \times 10^8$ CFU) | Inpatients | 46 (23/23) | 5 months–2 years | Viral (rotavirus) | Reduction in the number of episodes of diarrhea by 24.1% | 168 |
| *B. longum* PL03, *L. rhamnosus* KL53A and *L. plantarum* PL02 | $10^8$ CFU | Inpatients | 78 (40/38) | 5 months–16 years | AAD | No effects on diarrhea and associated symptoms | 218 |
| *L. rhamnosus* | $2 \times 10^{10}$ CFU | Inpatients | 240 (120/120) | 3 months–14 years | AAD | Reduction of diarrhea (by 11%) and AAD (6%) | 219 |
| LGG | $3.7 \times 10^{10}$ CFU | Outpatients | 204 (99/105) | 6 months–2 years | Bacterial, parasitic and viral | Reduction in the number of episodes of diarrhea by 13.45% | 156 |
| | $6 \times 10^9$ CFU | Inpatients | 81 (45/36) | 1 month–3 years | Viral (rotavirus) | Reduction in the incidence of nosocomial diarrhea by 26.6% | 220 |
| | $10^9$ CFU per 100 ml of fermented milk drink | Outpatients | 281 (139/142) | 13–86 months | Bacterial, parasitic and viral | No effects on diarrhea and associated symptoms | 221 |
| | $1 \times 10^9$–$1.2 \times 10^{10}$ CFU | Inpatients | 1092 | 1 month–18 years | Bacterial, parasitic and viral | Reduction in the incidence of diarrhea, including viral gastroenteritis | 222 |
| | $10^6$ CFU $mL^{-1}$ of Valio Gefilus milk | Outpatients | 231 (124/107) | 2–7 years | Bacterial, AAD | Reduction in antibiotic use, reduction in the number of gastrointestinal complaints and increase in the number of the beneficial microorganisms | 162 |
| LGG + micronutrients | $6 \times 10^9$ CFU $day^{-1}$ for 15 days | Inpatients | 90 (45/45) | 6 months–5 years | Bacterial, parasitic and viral (rotavirus; adenovirus) | Reduction in the incidence of diarrhea and hospitalization time (24 h) | 157 |
| *L. reuteri* DSM 17938 | $10^8$ CFU$day^{-1}$ | Inpatients | 106 (54/52) | 1 month–4 years | Viral | No effects on diarrhea and associated symptoms | 223 |
| | $1 \times 10^9$ CFU | Inpatients | 184 (91/93) | 1–48 months | Patients without previous diarrhea | No effects on diarrhea and associated symptoms | 155 |
| *S. boulardii* + antibiotics | 250 mg of *S. boulardii* | Inpatients | 269 (132/137) | 6 months–14 years | AAD | Reduction in the duration of diarrhea associated with antibiotics (used in the treatment of respiratory infections and otitis media) by 13.9% | 224 |
| Yogurt containing LGG, *B. lactis* (Bb-12) and *L. acidophilus* (La-5) | 200 g $day^{-1}$ | Inpatients | 70 (36/34) | 1–12 months | AAD | Reduction in antibiotic-associated diarrhea by 95.24% | 164 |

CFU: colony forming units. AAD: antibiotic-associated diarrhea. LGG: *L. rhamnosus* GG.

period of time (only 15 days). Besides, a total of 11/45 (24.4%) children taking this mixture had at least one episode of intestinal infection compared to 22/45 (48.9%) children in the placebo group ($P$ = 0.016).[157] Possibly, the supplementation with vitamins B and C and zinc accelerated the recovery of the children. Thus, the combination of micronutrients with probiotics represents a valuable and economical strategy for reducing or avoiding children's diarrhea, especially in low-income countries.

### 4.2. Antibiotic-associated diarrhea (AAD)

AAD is an important clinical syndrome in children worldwide, mainly due to excessive use of antibiotics in this age group, which causes them to be at a higher risk than adults.[158,159] *C. difficile* is the most frequent cause of AAD and is associated with greater severity.[159]

It was reported that 1 in every 7 cases of antibiotic-associated diarrhea is prevented by using probiotics.[160] A meta-ana-

lysis concluded that *S. boulardii*, LGG, *L. acidophilus* and *L. bulgaricus* reduce the occurrence of antibiotic-associated diarrhea by 57% (35–71%).[161] In addition, recent evidence demonstrated that the long-term LGG intake by preschool children prevents microbiota dysbiosis induced by penicillin treatment, increases the population of beneficial microorganisms including *Lactobacillus*, *Lactococcus*, *Prevotella*, and *Ruminococcus*, and reduces the use of antibiotics, such as macrolides and sulfonamide–trimethoprim.[162]

Of note, preventive studies on probiotic efficacy were performed in hospitalized patients and/or patients under antibiotic therapy. A recent review of 23 clinical trials suggests that probiotic intake may be beneficial against AAD in children; an effect that was observed in 22 out of the 23 evaluated trials.[163] Also, a yogurt containing LGG ($5.2 \times 10^9$ CFU $day^{-1}$), *B. lactis* (Bb-12; $5.9 \times 10^9$ CFU $day^{-1}$) and *L. acidophilus* (La-5; $8.3 \times 10^9$ CFU $day^{-1}$) was effective in reducing AAD in 95.24% of children, when administered from the start to the end of their antibiotic treatment.[164] In sum, the most recommended individual probiotic strains for the prevention of AAD in children, including *C. difficile* infections, are LGG and *S. boulardii* CNCM I-745.[159,165]

#### 4.3. Traveler's diarrhea

With regard to the role of probiotics in the treatment or prevention of traveler's diarrhea, the literature is divergent. A group of experts from the Asia-Pacific region considers the data to be insufficient and does not recommend the use of probiotics for children living in those countries who travel abroad.[166] On the other hand, the administration of *S. boulardii* to prevent traveler's diarrhea was associated with a significant reduction in its incidence in Latin America.[167] Thus, there is a need for additional randomized clinical trials to reach a conclusion based on stronger evidence.

## 5. Probiotic combinations

Some researchers have focused on the combination of various probiotic strains to potentiate their anti-diarrheal effects, although the achieved results are rather controversial. One of the first studies of the benefits resulting from the combined use of several probiotic microorganisms against intestinal infections was described more than 20 years ago.[168] A children's formula supplemented with *B. bifidum* and *S. thermophilus* promoted the reduction of the incidence of acute diarrhea among children aged 5 months to 2 years. Furthermore, a meta-analysis showed that the probiotics *L. acidophilus*, *L. bulgaricus*, LGG, and *S. boulardii* can reduce the risk of acute diarrhea for children by 57% used either alone or in combination.[161] By mixing probiotics (LGG, *B. lactis* (Bb-12) and *L. acidophylus* (La-5)) in yogurt, AAD was prevented in children although they still presented mild diarrhea and less adverse events, such as abdominal pain and vomiting.[164] However, another recent study did not detect any benefit in the length of hospital stays and diarrhea symptoms with the use of a mixture containing 80% *L. acidophilus*, 10% *L. bulgaricus*, 5% *B. bifidum* and 5% *S. thermophilus* as an adjuvant therapy for 290 children hospitalized with acute diarrhea.[169] This lack of effect might be due to the fact that the data were not obtained through a conventional randomized clinical trial. Interestingly, the combination of probiotics with antimicrobial agents was shown to be beneficial. Indeed, the combination of *S. boulardii* and metronidazole significantly reduced the duration of bloody diarrhea and contributed to a more efficient elimination of *E. histolytica* (6 days *versus* 5 days).[122]

## 6. Delivery of probiotics to children

As previously mentioned, single- or multistrain probiotics can be taken as additives in fermented dairy and non-dairy foods and beverages, as well as supplements in the form of tablets, capsules and freeze-dried preparations.[21–23,170,171] However, their usage in the clinic may depend on various factors intrinsic to the probiotic such as strain, resistance to the gastrointestinal tract, formulation, storage and viability, and intrinsic to the patient including age, disease and general condition.

Storage directly affects the efficacy of probiotics. Refrigeration is a major drawback in the use of probiotic foods or beverages, and although more stable, supplements and freeze-dried products need to be able to maintain high numbers of viable cell counts ($>10^7$ CFU $g^{-1}$ or CFU $ml^{-1}$) of each strain at least until their expiration date.[21] In this context, the formulation of probiotics into microcapsules can improve their survival and also control their release into the intestinal tract. In this system, probiotics are immobilized in a polysaccharide or protein matrix, which is resistant to the acidic pH of the stomach and thus, may boost the number of viable probiotic cells reaching the intestine.[172,173] However, more controlled studies are needed in order to determine which formulation is more efficient in delivering the highest numbers of viable probiotics able to stop diarrhea in children. Also, the efficacy of different probiotic microorganisms needs to be evaluated in the same delivery system.

When it comes to diarrhea in children, large amounts of food/beverage need to be consumed in order to treat the disease, which is not always possible for infants.[174] This limitation could be overcome by the use of supplements; however, formulations such as tablets or large capsules may not be suitable for very young children as they may be difficult to swallow. Interestingly, LGG ($10^{10–11}$ CFU per dose twice daily for five days) intake either in fermented milk or as freeze-dried powder was found to be equally effective in reducing the duration of diarrhea in children.[175] Therefore, the administration of freeze-dried products packed in sachets, associated or not with electrolytes for oral rehydration, or incorporated into specific infant formulas may represent an interesting approach for treating diarrhea in infants.[174]

Finally, the use of live or dead probiotics to treat disease has been debatable. While it has been well demonstrated in

 

the literature that different strains of probiotics are effective when given as live microorganisms, experimental findings in animals indicate that non-viable probiotics and their components (polysaccharides, membrane proteins, bacterial cell wall components, and even DNA) can also confer beneficial health effects, especially by modulating the immune response.[23,176] In this scenario, it has been suggested that non-viable probiotics may be applied to patients in certain clinical conditions such as immunodeficiencies, allergic diseases, and intestinal inflammation.[176] Although probiotic immunomodulatory properties are suggested to be retained by non-viable cells, this effect may not be immediate. Also, in non-viable formulations, the probiotic antimicrobial potential is lost. Thus, the administration of dead probiotics may not be as effective in treating acute diarrhea in children as formulations containing live cells.

## 7. Safety

In general, probiotic consumption is considered to be safe. However, there has been a major concern on their safety, especially in regard to reported cases of infections (bacteremia, sepsis, endocarditis and local infections) associated with probiotic-based products for the prevention and treatment of gastrointestinal disorders, which may arise from their repeated usage.[177–179]

*L. rhamnosus* was the most prevalent strain detected in these cases,[180,181] with reports including those in children with short gut syndrome.[178] In this case, bacteremia was observed in an 11 month old child who had received LGG ($1 \times 10^8$ CFU; twice daily) for 5 weeks for the treatment of rotavirus-related diarrhea.[178] Similarly, bacteremia was detected in newborns administered with $5 \times 10^9$ CFU of LGG (once or twice daily) to prevent AAD and necrotizing enterocolitis.[180] Also, cases of bacteremia associated with probiotics were found to be frequent in immunosuppressed (66%) and catheterized (82.5%) patients.[181] Sepsis was also observed in preterm infants fed with *L. rhamnosus* to avoid necrotizing colitis.[179] These negative effects were not observed in healthy-term infants ($n = 183$), as LGG ($10^6$ CFU) was found to be safe when administered in these individuals for as long as one year.[182] Similarly, the intake of $1 \times 10^8$ CFU $kg^{-1}$ $day^{-1}$ of *L. plantarum* by children and adolescents between 8 days prior to and 14 days after hematopoietic cell transplantation did not cause bacteremia or any serious adverse or unexpected effects.[109]

Other safety issues have been raised with probiotic use including the hypothesis that these bacteria may be able to transmit resistance genes to pathogenic and/or commensal microorganisms, although these pieces of evidence have been derived specially from *in vitro* studies with little knowledge of this event in humans.[183,184] It has also been suggested that probiotics might also cause gastrointestinal toxicity, especially in patients with short small bowel syndrome, probably by the accumulation of conjugated bile acid metabolites and subsequent malabsorption;[184] on the contrary, several studies have demonstrated a protective effect for different strains of probiotics in experimental models of intestinal cancer.[185,186]

These pieces of evidence suggest that probiotics should be given with caution to newborns or other individuals at risk, including those with immunosuppression.

## 8. Concluding remarks

Several clinical studies have investigated different strains, doses, routes and regimens of administration of probiotics. While diversification in the approach is crucial for more complete and dynamic analyses and outcomes, the multiplication of variables resulted in the current discrepancies among the results, even those obtained for the same probiotic strain but in different countries.[161] In addition, the beneficial effects exhibited by one probiotic strain cannot be extrapolated to all others, even those belonging to the same species.[187]

It is recommended that a product with probiotics and having health claims should contain at least $10^7$ CFU of viable probiotics per gram or ml.[33] However, several factors may affect the viability and efficacy of probiotics such as host factors, colonization properties of the probiotic strain, and manufacturing problems, among others. During passage through the gastrointestinal tract, the first barrier the probiotic must overcome is the extremely acidic pH of the stomach (ranging from 1 to 3) and a mean exposure time of approximately 1.5 hours. In the small intestine, the pH value increases to 5–6, but the bile salt concentration may reach 2% in the first hour of digestion and then decrease to 0.3% or less.[188] In this section, the intestinal transit may vary between 2.5 and 3 h.[189] So, to be considered as potential probiotics, the microorganisms are evaluated under conditions that simulate the gastrointestinal tract *in vitro*. Tolerance to these harsh conditions varies between strains of the same species and between different genera of microorganisms, but in general *Bifidobacterium* is less tolerant to acidic pH than *Lactobacillus*. On the other hand, several species of *Bifidobacterium* seem to be more resistant to high concentrations of bile salts. Therefore, since a large number of microorganisms may lose viability in the gastrointestinal tract, the microencapsulation of probiotics into gastroresistant capsules has been proposed to enable the gradual release of probiotics in the intestine and increase their resistance by protecting probiotic cells against adverse environmental conditions, principally in the upper portion of the gastrointestinal tract.[190]

Once administered, probiotics might or might not colonize the host's intestinal tract as a result of the different types of interactions between these microorganisms and the various possible gut microbiota profiles. Thus, probiotics might be inert or have beneficial actions or even pathogenic effects on individuals from different populations.[191] In addition, preparations containing insufficient numbers of viable probiotics and/or low purity may affect their efficacy. The analysis of the quality of nine probiotics sold in South Africa showed that only five of them contained viable bacteria in amounts

sufficient for a probiotic effect. The same study also showed that two of them were contaminated by *E. faecium*.[192] Moreover, labels providing inconsistent information were detected in almost 50% of commercialized probiotics.[193] All these problems may be related to poor surveillance by regulatory agencies. For this reason, the scientific community is strongly recommended to not only seek to discover "new probiotics" but also investigate the true properties and effects of several already commercialized products (by means of *in vivo* and clinical studies).[194,195]

On rare occasions, probiotics might cause some adverse effects, which might be severe in high-risk patients, such as immunosuppressed children. There have been reports of sepsis, fungemia, endocarditis and liver abscess caused by probiotic microorganisms.[177–179,196–199] Furthermore, probiotic microorganisms are potential carriers of resistance genes to pathogenic and opportunistic bacteria.[183] Although not as easily transferable as plasmids, some genes from certain *Lactobacillus* species confer natural resistance to vancomycin, and their spread to some bacteria, such as *Staphylococcus aureus* and the genus *Enterococcus*, is a cause of much concern.[183,200]

Another interesting point to highlight is the absence of clinical studies that take into account the effect of probiotics on the different diarrheagenic pathogens, which may trigger different degrees of severity. For example, EAEC, ETEC and *Shigella* produce potent toxins that mediate the intestinal secretion of fluids and electrolytes or the inhibition of protein synthesis culminating in watery diarrhea or bloody diarrhea with mucus, respectively.[201–203] The pathogenesis of *Salmonella* spp. and *Shigella* spp. include the invasion of small or large intestinal enterocytes which cause extensive inflammatory changes that can progress to an ulcer.[204,205] Also, in the case of viral diarrhea, probiotic use was shown to be controversial, and treatment outcome may depend not only on the pathogen causing the disease, but also on the probiotic used as a treatment. This is suggested by evidence that LGG,[130,140] but not *L. paracasei*,[146] was beneficial against diarrhea, and further supported by a study comparing the effects of *S. boulardii* and a combination of *L. acidophilus*, *L. rhamnosus*, *B. longum* and *S. boulardii* on children, which showed that these probiotics differently affect the symptoms of disease (diarrhea, vomiting and fever).[206]

Another possible approach to end the controversies on the use of probiotics is to prepare population-specific or even personalized formulas. New probiotics might come to be designed based on the microorganisms isolated from the endogenous gut microbiota in order to be administered to individuals belonging to the same population from which they were isolated.[191] For this purpose, well-designed multicenter studies are needed to previously assess distinct gut microbiome profiles and to correlate it with the possible effects of the microorganisms that will be administered.

Discrepancies notwithstanding, both clinical studies and meta-analyses reported that the probiotics most studied up to the present time (LGG, *L. acidophilus*, *L. reuteri* and *S. boulardii*) shorten the duration of diarrhea and the length of hospital stays by 24 hours.[207,208] Although this time reduction is not extensive, it is associated with considerable socioeconomic benefits due to reductions in hospital costs, eventual avoidance of unnecessary antibiotic therapy and promotion of faster patient recovery. Among all of the studied probiotics, LGG and *S. boulardii* exhibited the most consistent evidence and, thus, may be used with greater safety in routine pediatric care.[207] All other microorganisms still need to be more thoroughly assessed in randomized, double-blind studies, at different doses and with different populations.

*In vivo* and clinical studies should more thoroughly assess the effects of probiotics on a wide range of clinical forms of infectious diarrhea and investigate the mechanisms of action and responses elicited by the interactions of these microorganisms with various pathogens and their variable virulence factors. It is worth stressing that most clinical studies of probiotics are conducted in developed countries, while children from developing and, more specifically, under-developed countries might exhibit completely different responses to these microorganisms. However, according to a WHO survey, the incidence of childhood diarrhea (less than 5 years old) does not differ significantly when compared among the different regions of the world, but the most severe cases and mortality are much higher in low-income countries than in middle- and high-income countries. The majority of severe cases of diarrhea in the world occurred principally in Africa (26.6%) and southeast Asia (25.8%), but in other regions the occurrence of severe diarrhea is still relevant, including countries located in the following WHO regions: Western Pacific (15.2%), Americas (13.3%), Eastern Mediterranean (11.9%), and Europe (6.9%%), with a total of more than 36 million severe episodes estimated in 2010.[209]

Further investigations using modern sequencing technologies, *i.e.*, next-generation sequencing, to obtain safer and more reliable results on the composition and evolution of the gut microbiome before and after the use of probiotics are needed. This approach should be correlated with clinical results to achieve a better understanding of the role each probiotic strain plays in the restoration and maintenance of the gut microbiota balance.

Another important but still unknown issue concerns the impact that long continuous ingestion of probiotics would have on the intestinal ecosystem of healthy individuals. It is well known that ingestion of different probiotic strains has not been associated with long-term colonization in the host, since probiotic strains are only maintained transiently for days after the patients discontinued their ingestion.[210,211] Therefore, their effects appear to be transient and thus require continued long-term consumption. One may argue whether prolonged use could not be a kind of selective pressure on other microorganisms in a manner similar to the indiscriminate use of antibiotics. If this is a concrete possibility, it would lead to the ineffectiveness of probiotics in relation to their antimicrobial activity. Although current evidence does not indicate loss of beneficial organisms from the microbiome, questions regard-

 

ing the long-term effects of probiotic ingestion on otherwise healthy individuals remain unanswered. Further research is needed to clarify whether healthy individuals benefit from the regular intake of probiotics.

## Conflicts of interest

There are no conflicts to declare.

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4.2 CAPÍTULO 2 - Antimicrobial activity of *Bifidobacterium* and *Lactobacillus* species from children´s feces against bacterial enteropathogens (submetido à revista Anaerobe, Qualis B1 em Medicina I e Fator de Impacto 2.742).

**Antimicrobial activity of *Bifidobacterium* and *Lactobacillus* species from children´s feces against bacterial enteropathogens**

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**ABSTRACT**

Diarrhea accounts for over 500,000 deaths annually among children under the age of five years. Traditional approaches to treatment, such as electrolyte replacement and antibiotic therapy, sometimes are insufficient to remedy all the deleterious effects caused by enteric pathogens. In this scenario, the use of probiotic microorganisms has been considered an innovative therapeutic option for treating such infections. This study sought to investigate the probiotic potential of microorganisms, *namely Bifidobacterium and Lactobacillus,* isolated from the feces of healthy children. *Bifidobacterium* and *Lactobacillus* present in stool samples of 14 healthy children were quantified by plate counting in MRS agar and identified by PCR with genus-specific primers. Bacterial isolates with mucin-binding ability were further identified at species level by 16S rRNA sequencing. Then, their probiotic potential was assessed in tolerance assays to determine resistance to gastrointestinal cues such as lysozyme, acidic pH, and bile salts. Also, the ability of these probiotic bacteria to inhibit growth of enteropathogens and pathogen adhesion to mucus was investigated. *B. animalis* subsp. *lactis* L56.1, *B. bifidum* A14.2, *B. longum* C25.3, *B. longum* N49.3, and *L. fermentum* L54.2 survived the gastrointestinal conditions ($p < 0.001$) tested, bound mucin, and inhibited enteropathogen growth and their binding to mucin. Overall, the data highlight the potential of these microorganisms as probiotics as they inhibited the main bacterial pathogens associated with moderate and severe childhood diarrhea, thus, representing an interesting strategy for the treatment of these pathologies.

**Keywords:** probiotics; *Bifidobacterium*; *Lactobacillus*; enteropathogens; diarrhea.

## 1. Introduction

According to the United Nations International Children's Emergency Fund (UNICEF), diarrhea was the second major communicable cause of death among children under five years of age, resulting in approximately 480,000 deaths in 2016. In general, children residing in the less developed regions of the world are the most frequently affected [1]. Many cases of diarrheal diseases are caused by viruses; however, an increasing number of bacterial diarrhea cases has been reported [2]. In this context, diarrheagenic *Escherichia coli* (DEC), *Salmonella* spp., and *Shigella* spp. are among the most prevalent agents of bacterial infections worldwide [3-11].

In general, diarrhea treatment comprises the prevention of dehydration by oral or intravenous rehydration solutions [12], although this therapy is unable to reduce the duration of diarrhea and stool frequency or to restore the gut microbiota [12, 13]. Anti-diarrheal drugs, such as loperamide and diosmectite, which promote reduction of intestinal motility have also been used, but their benefits have been inconsistent [14, 15]. On the other hand, antimicrobials should only be prescribed to treat specific causes of diarrhea, such as cholera, *Shigella* dysentery, amebiasis, and giardiasis [12].

The use of probiotics in the treatment of diarrheal diseases has been investigated; however, there are still controversies about their efficacy for the control or prevention of infectious diarrhea [16], as they may be species-specific or even, strain-specific [17-19]. Also, reductions in the disease period of only 24 h have been reported after the use of some probiotic strains [16 86]. Thus, there is a need to identify new strains with a more targeted antimicrobial action against the most relevant enteric pathogens, which may be able to confer greater benefits to patients.

Currently, *Bifidobacterium* and *Lactobacillus* species are among the most commonly used probiotics, commercially available either alone or in combination with various

other species [20, 21]. As large-scale production of probiotics has already been optimized, products comprising these probiotic species have a long-term shelf life [22].

In this study, a screening for potential *Bifidobacterium* and *Lactobacillus* probiotic strains was performed in isolates obtained from stool samples of children without intestinal disorders. These were evaluated for their ability to tolerate harsh gastrointestinal tract conditions, to bind to mucin, and to inhibit growth and mucin binding activity of enteropathogens. Our findings highlight the potential of the probiotic *Bifidobacterium* and *Lactobacillus* isolates found here in the treatment or prophylaxis of gastrointestinal diseases.

## 2. Materials and methods

### *2.1. Growth conditions of bacterial strains*

The following enteropathogenic bacteria were used: enteroaggregative *E. coli* 042 (EAEC), enterohemorrhagic *E. coli* EDL933 (EHEC), enteropathogenic *E. coli* E2348/69 (EPEC), enterotoxigenic *E. coli* 1661-1 (ETEC), *Salmonella enterica* serotype Choleraesuis INCQS 028, and *Shigella flexneri* 2a. The strains were grown and maintained in brain heart infusion (BHI) broth (Difco-BD, Detroit, MI, USA) under aerobic atmosphere at 37 °C for 24 h. Isolates of *Bifidobacterium* and *Lactobacillus* were grown in de Man, Rogosa, and Sharpe medium (MRS broth and agar, Difco-BD) containing 0.25% L-cysteine and incubated at 37°C for 48 h under anaerobic conditions. *Bifidobacterium longum* subsp. *longum* ATCC 15707 and *Lactobacillus fermentum* ATCC 23271 were used as positive controls in all experiments. In addition, replicates of all bacterial strains were maintained at -80°C in MRS broth or BHI with 20% glycerol.

### *2.2. Sampling of participants and feces collection*

Fourteen children aged 1 month to 5 years who attended the Dr. Odorico Amaral de Matos Children's Hospital for reasons other than diarrhea or other intestinal disorders and who did not receive antibiotics in the last 30 days prior to sample collection were randomly enrolled in the study. All mothers were instructed to collect the children's feces with the aid of a sterile swab. Specimens were placed into a sterile collector, and immediately processed for the isolation of *Bifidobacterium* and *Lactobacillus* strains. The parents or legal guardians of the participating children were informed on the nature of the research and signed the "Free and Informed Consent Form". The study protocol was approved by the Research Ethics Committee of the CEUMA University (No. 791.457) and authorized by the Municipal Department of Health (São Luís, Maranhão, Brazil).

*2.3. Bacterial plate counting*

To determine *Bifidobacterium* and *Lactobacillus* total bacterial numbers, fecal samples were weighted and serially diluted in phosphate-saline buffer PBS (pH 7.2, Sigma-Aldrich, St. Louis, MO) up to $10^{-8}$ dilution. Aliquots of 0.1 mL of each dilution were spread onto MRS agar (Difco-BD) containing 0.25% L-cysteine, and incubated at 37°C for 48 h under anaerobic conditions (Anaerobac system, Probac®, São Paulo, Brazil). After incubation, the colony morphotypes were subjected to Gram staining and the catalase test. Suspicious colonies of Gram-positive and catalase-negative bacilli were counted and identified by PCR using genus-specific primers. Colonies were stored in MRS broth containing 20% glycerol at -80°C for further analyzes [23].

*2.4. Molecular identification of* Bifidobacterium *and* Lactobacillus

Bacterial DNA was extracted from candidate bacilli strains after growth in MRS broth by using MagaZorb® DNA Mini-Prep Kit (Promega, Madison, WI-USA) according to

the manufacturer's instructions. Polymerase chain reaction (PCR) amplification was performed using *Lactobacillus*-specific genus primers: Lacto-16S-FW (5′-GGAATCTTCCACAATGGACG-3′) and Lacto-16S-RV (5′-CGCTTTACGCCCAATAAATCCGG-3′) [24] and *Bifidobacterium*-specific genus primers: Bif164 (5′-CATCCGGCATTACACCGGGAA-3′) and Bif662 (5′-CCACCGTTACACCGGGAA -3′) [25]. Bacterial isolates positive for one the genus-specific primer sets were identified at species level by 16S rRNA sequencing using the 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) universal primers [26]. The amplicons were purified by using the Wizard SV Gel and PCR Clean-up System (Promega) and were sequenced in both sense and anti-sense directions at the Myleus Biotechnology Facilities (Belo Horizonte, MG, Brazil). Sequences were compared with other sequences deposited in GenBank, using the program BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignments were generated using the program ClustalW [27] implemented by the software Mega 6 (Molecular Evolutionary Genetics Analysis, version 6.0) [28]. Alignments were corrected manually and the analysis was carried out using the Mega 6 software.

*2.5. Tolerance to acid pH, lysozyme, and bile salts*

To mimic some of the gastrointestinal tract harsh conditions to which these bacteria are normally exposed, assays to determine tolerance to acidic pH, lysozyme, and bile salt were performed as previously described [29]. Initially, suspensions of microorganisms belonging to the genera *Bifidobacterium* and *Lactobacillus* were adjusted to the optical density value of 0.1 ± 0.01 at 600 nm (~1 $\times 10^8$ CFU/mL) in MRS broth. Then, 96-well plates containing modified MRS medium were prepared under the following conditions, independently: (1) supplemented with 300 μg/mL lysozyme (Sigma-Aldrich); (2)

adjusted to pH 2 and pH 4, and (3) supplemented with 0.5% and 1% bile salts (Difco-BD). Tolerance assays were performed after the addition of 100 µL of the standard bacterial suspensions in the 96-well plates containing 100 µL of the modified MRS medium prepared under the conditions described above. The plates were then incubated at 37°C under an anaerobic atmosphere for 6 h. In parallel, serial decimal dilutions of the assays were performed at different time-points (0-360 min) to determine the number of microorganisms grown on MRS agar containing 0.25% cysteine. Wells with non-adjusted MRS medium with and without inoculated bacteria were used as positive and negative controls, respectively.

*2.6. Binding to mucin*

This assay was performed to determine the ability of *Bifidobacterium*, *Lactobacillus* and enteropathogenic strains to bind swine gastric type III mucin (Sigma-Aldrich). Initially, mucin was solubilized in PBS (pH 7.4) at a final concentration of 10 mg/mL. Five hundred microliters of the bacterial suspension ($1.5x10^8$ CFU/mL) were added to each well of the 24-well plates, which were then, incubated overnight at 4°C. After incubation, the wells were washed three times with 1 mL of PBS (pH 7.4) and saturated with 2% bovine serum albumin (Cohn V fraction) (INLAB, São Paulo, Brazil). The plates were then incubated for 4 h at 4°C. After incubation, the plates were washed four times with 1 mL PBS (pH 7.4) to remove albumin residues. Then, the bacterial suspensions (*Bifidobacterium*, *Lactobacillus* and enteropathogens) were centrifuged at 5,000 x *g* for 10 min, washed three times with PBS (pH 7.4), and the pellets were resuspended in PBS (pH 7.4), adjusted to $10^8$ CFU/mL. Aliquots (500 µL) of the bacterial suspensions were added to each well and the plates were incubated at 37 °C for 3 h. After incubation, the wells were washed three times with 1 mL PBS (pH 7.4) to eliminate non-adherent bacteria. Subsequently, the wells were treated with 200 µL of

146 0.5% Triton X-100 for 10 min and subjected to tip scraping. Quantification of bacteria
147 adhered to mucin was performed by inoculating MRS agar with 100 µL aliquots of the
148 bacterial suspensions after decimal serial dilutions ranging from $10^{-1}$ to $10^{-5}$. Mucin-
149 containing wells without bacteria were used as negative controls [30].

150 *2.7. Inhibition of enteropathogen binding to mucin*

151 After 3 h incubation of enteropathogens with mucin, suspensions of *Bifidobacterium*
152 and *Lactobacillus* ($10^8$ CFU/mL) were added to the wells and incubated at 37 °C for
153 additional 3 h, as described above. Quantification of the inhibition of pathogen binding
154 to mucin was performed as described above. Mucin-containing wells without bacteria
155 were used as negative controls [31].

156 *2.8. Antimicrobial activity*

157 The antimicrobial activity of *Bifidobacterium* and *Lactobacillus* isolates against
158 enteropathogens was assessed as previously described by Mastromarino et al. (2002)
159 with minor modifications. For this assay, overnight cultures of each isolate were
160 centrifuged at 5,000 x *g* for 10 min and the pellets were washed three times with PBS
161 (pH 7.4). Then, they were resuspended in 1 mL of PBS (pH 7.4) and adjusted to an
162 optical density value of 0.1 ± 0.01 at 600 nm (~$1 \times 10^8$ CFU/mL). Aliquots of 10 µL
163 were spotted on the surface of MRS agar plates (90 × 15 mm) and incubated at 37 °C
164 for 24 h under anaerobic conditions. Then, 10 mL of Mueller-Hinton agar (Difco-BD) at
165 50 °C were overlaid onto the MRS agar plate, which contained the circular growth areas
166 of each test isolate. After solidification at room temperature (25 – 28 °C), adjusted
167 suspensions ($1.5 \times 10^8$ CFU/mL) of each enteropathogen were spread onto the surface
168 of the agar with the aid of cotton swabs and the plates were incubated at 37 °C for 24 h
169 under aerobic atmosphere. Antimicrobial activities were characterized by clear zones of

growth inhibition around the *Bifidobacterium* and *Lactobacillus* growth areas expressed in millimeters [32].

### *2.9. Statistical analyses*

GraphPad Prism (version 5.0; GraphPad Software, Inc. San Diego, CA, USA) software was employed for statistical analysis. The normality of the assays was evaluated by the Kolmogorov-Smirnov test, followed by repeated measures of variance analysis (ANOVA) for mucin adhesion and inhibition of mucin adhesion tests for tolerance to lysozyme, pH, and bile salts. The Dunnett's test was employed to compare different groups of microorganisms with respect to the control. Statistical significance was established at $p < 0.05$, $p < 0.01$, and $p < 0.001$. All assays were performed in triplicate on three independent days.

## 3. Results

### *3.1. Quantification of* Bifidobacterium *and* Lactobacillus *in children's feces*

Bacterial quantification in feces is shown in Table 1. Fecal samples of 13 children *Lactobacillus* presented counts from $10^5$-$10^9$ CFU/g, whereas only 5 had *Bifidobacterium* counts ≥ $10^5$ CFU/g (up to $10^8$ CFU/g). Forty-one colonies of Gram-positive and catalase-negative microorganisms amplified DNA fragments with genus-specific primers and were selected for further analysis, of which 32 were identified as *Lactobacillus* and 9 as *Bifidobacterium* by PCR.

### *3.2. Binding to mucin*

The 41 Gram-positive isolates were tested for their ability to bind mucin. Of these, only 10 were classified as adherent because they had a similar or higher adhesion pattern than *L. fermentum* ATCC 23271 and *B. longum* subsp. *longum* ATCC 15707, which

were included as positive controls (Fig. 1). Among the adherent microorganisms, two *Bifidobacterium* isolates (strains C25.3 and S34.10) exhibited higher adhesion ($p < 0.001$ and $p < 0.05$, respectively) in comparison with the control microorganisms.

### *3.3. Bacterial identification by 16S rRNA sequencing*

The 10 mucin-adherent isolates were selected for identification by 16S rRNA sequencing. The sequence analysis of nine isolates using the BLAST program showed 99.16–100% sequence homology with known species. Three isolates (S34.1, S34.10, and L56.1) were identified as *Bifidobacterium animalis* subsp. *lactis*, two (A14.2 and S34.5) as *Bifidobacterium bifidum*, one (R32.2) as *Bifidobacterium breve*, two (N49.3 and C25.3) as *Bifidobacterium longum*, and one (L54.2) as *Lactobacillus fermentum*. The M24.2 was the only isolate to show a sequence homology of less than 97% with *Lactobacillus brevis* (Table 2).

### *3.4. Tolerance to pH, lysozyme, and bile salts*

Tolerance assays for acidic pH (2 and 4), lysozyme, and bile salts (0.5% and 1%) were performed to evaluate the resistance of the isolates to these gastrointestinal tract traits. Among the 10 isolates, only *B. animalis* subsp. *lactis* S34.10 and *L. brevis* M24.2 were not resistant to pH 2 and 1% bile salts, respectively (Table 3, Supplementary Fig. 1–4). Both isolates were excluded of further assays.

### *3.5. Antimicrobial activity assay*

The 8 isolates selected after the mucin binding and tolerance assays were further tested for their antimicrobial activity against DEC strains, *S. enterica* serotype Choleraesuis, and *S. flexneri*, which was based on the formation of clear zones of growth inhibition of enteropathogen around the *Bifidobacterium* and *Lactobacillus* growth areas (Fig. 2). The isolates presented antimicrobial activity against all the enteropathogens tested, but

with zones of growth inhibition with diameters varying from 9 to 25 mm (Table 4). EHEC EDL933 and EPEC E2348/69 were found to be the most sensitive pathogens to the antimicrobial action of the potential probiotics.

### *3.6. Displacement inhibition assay*

Isolates that produced inhibition zones of ≥ 10 mm against all enteropathogens as determined by the antimicrobial activity assays were tested for their ability to inhibit binding to mucin, including *B. animalis* subsp. *lactis* L56.1, *B. bifidum* A14.2, *B. longum* C25.3, *B. longum* N49.3, and *L. fermentum* L54.2. Notably, *Bifidobacterium* and *Lactobacillus* species inhibited significantly the mucin-binding activity of all the enteric pathogens tested ($p < 0.001$), with percentages of inhibition ranging from 35–100% (Fig 3).

## 4. Discussion

This study sought to investigate the probiotic potential of microorganisms isolated from the feces of healthy children. In particular, *Bifidobacterium* and *Lactobacillus* species have been looked at as intestinal microorganisms that possess probiotic activity and thus their use in the treatment or prophylaxis of intestinal diseases associated to bacterial pathogens is promising. *Bifidobacterium* and *Lactobacillus* are the most prevalent bacterial genera in the gut microbiota of healthy infants [33]. We began the present study by determining the presence of these two bacterial species in stool samples of 14 healthy using conventional bacteriological and PCR-based techniques. The bacterial isolates were tested for mucin-binding ability, tolerance to lysozyme, acidic pH, and bile salts. Lastly, their ability to inhibit the growth and adhesion of bacterial pathogens to mucus was investigated.

We found that the level of bacterial counts of *Lactobacillus* in children's stools was higher than that of *Bifidobacterium*. Differences in the population of these organisms among children between this and previous studies may be due to population intrinsic differences (i.e., the age range of the children selected for the study, type of feeding, the mode of delivery, immune system, among others). These early-life events modulate the intestinal microbiota and modify the microbial succession of *Bifidobacterium*, *Lactobacillus*, and *Bacteroides* with species-specific differences over time [34]. Also, variations in bacterial culturing approaches by different laboratories may account for the discordant amounts of *Bifidobacterium* and *Lactobacillus* found in the present study. We used only MRS medium with 0.25% cysteine that is ideal for the recovery of *Lactobacillus*, but it may not be enough for the isolation of all species of *Bifidobacterium.* The use of broader approaches of culture conditions is critical for the recovery of fastidious and anaerobic microorganisms and are directly related to the diversity and complexity of the microorganisms studied [35].

*Bifidobacterium* isolates had a greater binding capacity to mucin. In particular, *B. animalis* subsp. *lactis* S34.10 and *B. longum* subsp. *longum* C25.3 showed higher adhesion to mucin when compared to the control strain *B. longum* subsp. *longum* ATCC 15707 (Fig. 1). Binding to mucin is commonly used for the selection of probiotic microorganisms, as it is an indicative criterion for their potential ability of host colonization [36]. However, this interaction is complex, multifactorial, and a strain-specific character because of the diversity of exopolysaccharides [37] and protein components that are present in intestinal mucus [38-40].

On the hand, among the *Lactobacillus* isolates found, *L. brevis* M24.2 and *L. fermentum* L54.2 were also prominent in their interaction with mucin and they exhibited similar adherence profiles to those observed for *L. fermentum* ATCC 23271, used herein as a

positive control. Many *Lactobacillus* components have been described as putative colonization factors, including glyceraldehyde-3-phosphate dehydrogenase in *L. plantarum* BMCM12 [41], GroEL in *L. johnsonii* La1 [42], fibronectin-binding protein in *L. acidophilus* NCFM [43], mucin-binding protein in *L. fermentum* 104R [44], the RLBRO1264 protein of *L. brevis* ATCC 367 [45], and the elongation factor Tu in *L. johnsonii* NCC533 [46]. These data indicate that distinct species and even strains of *Lactobacillus* can interact with the host by using different adhesive factors.

The mucin-binding *Bifidobacterium* and *Lactobacillus* spp. were analyzed for their capacity to tolerate conditions like those found at the gastrointestinal tract. Lysozyme is found in biological fluids such as saliva (75 μg/mL), mucus, and breast milk (17-184 μg/mL) [47-50], and degrades peptidoglycans of Gram-positive bacteria. The mechanisms of *Bifidobacterium* resistance to lysozyme remain unknown; however, there is a variation in lysozyme susceptibility that is strain-specific [51]. In general, all the microorganisms studied here showed tolerance to 300 μg/mL of lysozyme, a concentration which is greater to the concentration of lysozyme found in body fluids.

During the transition from the stomach to the small intestine, microorganisms encounter a second biological barrier, i.e., a pH gradient, which ranges from 2 to 6 [52]. Lactic acid bacteria can resist acidic pH because their F0F1-ATPase enzyme can directly pump $H^+$ protons into the cytoplasm [53]. In fact, bacteria may be able to withstand a pH range from 2 – 3 for a period of 3 h, which are considered gastric acid-tolerant [54]. Except for *B. animalis* subsp. *lactis* S34.10, the other *Bifidobacterium* and *Lactobacillus* isolates could tolerate acid stress, which is in accordance with previous findings [55, 56]. The adaptive mechanism of *B. longum* to acidic pH is related to alterations in glycolytic flow and the ability of the microorganism to regulate its internal pH [57].

As tolerance to the acidity of the gastrointestinal tract is a relevant factor for bacterial survival, resistance to bile salts at 0.15% and 0.3% is also an important criterion for the selection of probiotic microorganisms for human use [56, 58]. In this context, the significant multiplication of *Bifidobacterium* and *Lactobacillus* isolates observed in the presence of 0.5% and 1% bile salts indicate that these bacteria may be able to utilize bile salts in their metabolism. The ability to metabolize bile salts is an important factor for the survival of several commensal and pathogenic microorganisms in the intestinal tract [59]. The active efflux and hydrolysis of bile acids [60, 61], [62, 63], and the changes in the architecture/composition of the cell membrane and cell wall [55, 64] confer resistance to bile among bacteria belonging to these genera.

After crossing gastrointestinal barriers and adhesion to mucus, the probiotic microorganisms must compete with resident bacteria. *Lactobacillus* and *Bifidobacterium* can inhibit other microorganisms by secreting a variety of antimicrobial compounds, such as organic acids, hydrogen peroxide, bacteriocins, and biosurfactants, which may act *per se* or synergistically [20, 65]. In the present study, the combined action of these antimicrobial compounds was evaluated by the agar diffusion test. We found that all the selected *Bifidobacterium* and *Lactobacillus* species reduced the growth of the pathogens evaluated. In this context, lactic acid bacteria and *Bifidobacterium* have shown a broad spectrum of action against food contaminating pathogens, including *E. coli*, *Salmonella*, and *Shigella* [66].

It was recently reported that *Lactobacillus rhamnosus* GG inhibits growth of *Salmonella* Typhimurium by promoting the accumulation of lactic acid in the cytoplasm of the pathogen and the collapse of the proton motive force, which prevented the transport of nutrients [67]. The dissociated forms of organic acids can penetrate the outer membrane

313 of gram-negative bacteria and reduce the internal pH, destroying acid-sensitive
314 microorganisms [68].

315 In the present study, the wild-type strain *L. fermentum* L54.2 showed a greater spectrum
316 of action against pathogens. We have recently reported the antimicrobial action of *L.*
317 *fermentum* ATCC 23271 against genital pathogens, including clinical strains of *Candida*
318 spp. associated with vaginitis in women [31]. Of note, the antimicrobial action of *L.*
319 *fermentum* has been associated with the secretion of bacteriocins, such as fermenticin
320 HV6b [69]. All these data suggest that this *Lactobacillus* species may have an intrinsic
321 antimicrobial activity.

322 In addition, another relevant finding was the inhibition of enteropathogen binding to
323 mucin displayed by *B. bifidum* A14.2, *B. longum* C25.3, *B. longum* N49.3, *B. animalis*
324 subsp. *lactis* L56.1, and *L. fermentum* L54.2. It is important to mention that bacterial
325 colonization of host tissues is a central event for the establishment of an intestinal
326 infection [70, 71] and that the gut epithelium is covered by mucus [72]. Also,
327 *Bifidobacterium* and *Lactobacillus* isolates inhibited the adhesion of enteropathogens
328 with distinct mechanisms of pathogenesis, indicating that such probiotic candidates may
329 have great potential as a commercial perspective. An interesting alternative would be
330 the development of a probiotic composed of several species.

331 **6. Conclusion**

332 Several criteria are considered when proposing the use of microorganisms as potential
333 probiotics, including their viability in the gastrointestinal tract and ability to act at the
334 site of interest [73]. Based on them, the fecal isolates *B. bifidum* A14.2, *B. longum*
335 C25.3 and N49.3, *B. animalis* subsp. *lactis* L56.1, and *L. fermentum* L54.2 exhibited
336 tolerance to acidic pH ranges, high concentrations of bile salts, and lysozyme, as well as

inhibited enteropathogen growth and binding to mucin. These properties can confer selective advantages and desirable characteristics for their use as probiotics in the treatment of diarrhea in humans. However, future studies are needed to evaluate their efficacy in animal models and clinical trials, as well as their safety for human oral administration.

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## Conflict of Interest disclosure

The authors have no conflicts of interest to declare.

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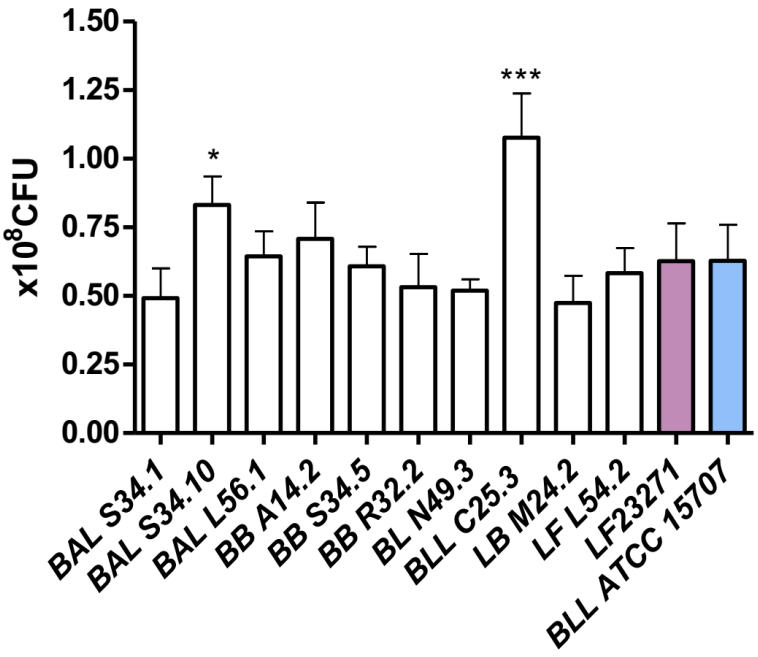


**Figure 1.** Quantification of mucin adhesion by *Lactobacillus* and *Bifidobacterium* spp. The assay was performed in gastric mucin of swine type III. Quantification of bacteria adhered to mucin was performed by plating decimal serial dilutions onto MRS agar of the bacterial suspensions. Mucin-containing wells without bacteria were used as negative controls. BAL S34.1: *B. animalis* subsp. *lactis* S34.1; BAL S34.10: *B. animalis* subsp. *lactis* S34.10; BAL L56.1 = *B. animalis* subsp. *lactis* L56.1; BBF A14.2 = *B. bifidum* A14.2; BBF S34.5 = *B. bifidum* S34.5; BB R32.2 = *B. breve* R32.2; BL N49.3 = *B. longum* N49.3; BLL C25.3 = *B. longum* subsp. *longum* C25.3; LB M24.2 = *L. brevis* M24.2; LF L54.2 = *L. fermentum* L54.2. The strains *L. fermentum* ATCC 23271 (LF23271) and *B. longum* subsp. *longum* ATCC 15707 (BLL15707) were used as positive controls. The assays were performed in triplicate. CFU= colony forming units. *$p < 0,05$; **$p < 0,001$.

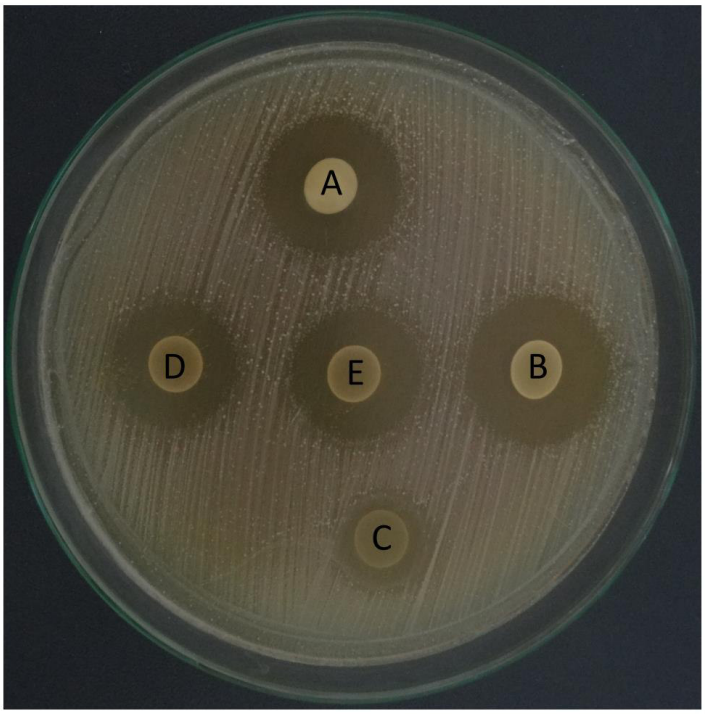


**Figure 2.** Representative result of the antimicrobial activity assay. Aliquots of adjusted suspensions ($10^8$ CFU/mL) of *Bifidobacterium* and *Lactobacillus* were spotted on MRS agar plates and incubacted at 37 °C for 24 h under anaerobic atmosphere. Then, 10 mL of Mueller-Hinton agar at 50 °C were overlaid onto the MRS agar plate. After solidification, adjusted suspensions ($1.5 \times 10^8$ CFU/mL) of each enteropathogen were spread on the surface of the agar with the aid of cotton swabs and the plates were incubated at 37 °C for 24 h under aerobic atmosphere. EAEC 042. Legend: A) *L. fermentum* ATCC 23271, B) *L. fermentum* L54.2, C) *B. bifidum* S34.5, D) *B. longum* N49.3, and E) *B. longum* ssp. *longum* ATCC 15707.

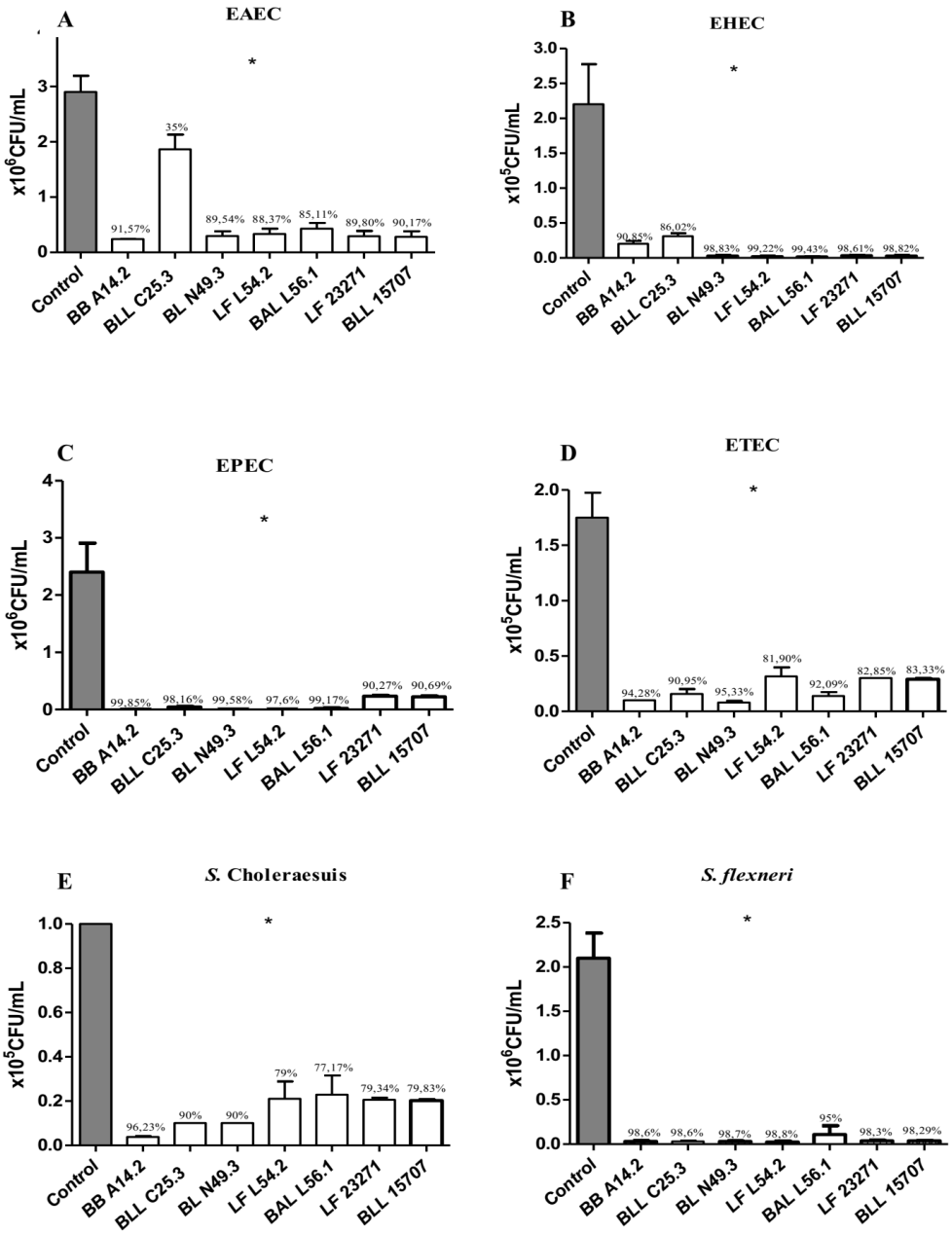


**Figure 3.** Inhibition of enteropathogens binding to mucin by *Bifidobacterium* and *Lactobacillus* isolates. Quantification of bacteria adhered to mucin was performed by plating decimal serial dilutions onto MRS agar. Mucin-containing wells without bacteria were used as negative controls. Enteroaggregative *E. coli* 042 (EAEC), enterohemorrhagic *E. coli* EDL933 (EHEC), enteropathogenic *E. coli* E2348/69 (EPEC), enterotoxigenic *E. coli* 1661-1 LT/ST +(ETEC), *S. enterica* serovar Choleraesuis INQS 028 (*S.* Choleraesuis), and *S. flexneri* 2a (*S. flexneri*). All assays were performed in triplicate. CFU= Colony forming units. * $p$ <0,001.

668 **Table 1**

669 Quantification of *Bifidobacterium* and *Lactobacillus* in children's feces

| **Patient ID No.** | **CFU/g*** | |
|---|---|---|
| | ***Bifidobacterium*** | ***Lactobacillus*** |
| 14 | $2 \times 10^5$ | $< 10^5$ |
| 21 | $< 10^5$ | $2{,}3 \times 10^9$ |
| 24 | $< 10^5$ | $5{,}5 \times 10^7$ |
| 25 | $3 \times 10^8$ | $1{,}5 \times 10^5$ |
| 32 | $2{,}5 \times 10^8$ | $1 \times 10^7$ |
| 34 | $< 10^5$ | $1 \times 10^8$ |
| 46 | $1{,}5 \times 10^8$ | $5 \times 10^8$ |
| 49 | $< 10^5$ | $1{,}6 \times 10^8$ |
| 54 | $5{,}5 \times 10^8$ | $1 \times 10^8$ |
| 56 | $< 10^5$ | $2{,}5 \times 10^8$ |
| 64 | $< 10^5$ | $1{,}1 \times 10^9$ |
| 85 | $< 10^5$ | $1 \times 10^8$ |
| 86 | $< 10^5$ | $2 \times 10^7$ |
| 87 | $< 10^5$ | $1 \times 10^7$ |

670 * Quantification was expressed as CFU/g or colony forming units per
671 gram. Gram-positive and catalase-negative bacilli were identified as
672 *Bifidobacterium* or *Lactobacillus* by PCR with genus-specific primers.

673

674 **Table 2**

675 Species identification by 16S rRNA sequencing of 10 bacterial isolates from children's

676 feces.

| Isolates | Species designation | Homology (%) | No. access |
|---|---|---|---|
| BAL S34.1 | *Bifidobacterium animalis* ssp. *lactis* | 99.79 | MK561772 |
| BAL S34.10 | *B. animalis* ssp. *lactis* | 100.00 | MK561773 |
| BAL L56.1 | *B. animalis* ssp. *lactis* | 99.79 | MK561774 |
| BBF A14.2 | *Bifidobacterium bifidum* | 99.93 | MK561775 |
| BBF S34.5 | *B. bifidum* | 99.93 | MK561776 |
| BB R32.2 | *Bifidobacterium breve* | 99.92 | MK561777 |
| BL N49.3 | *Bifidobacterium longum* | 99.93 | MK561778 |
| BLL C25.3 | *B. longum* | 100.00 | MK561779 |
| LB M24.2 | *Lactobacillus brevis* | 96.09 | MK561780 |
| LF L54.2 | *Lactobacillus fermentum* | 99.16 | MK561781 |

677

678

679 **Table 3**

680 Tolerance assays to adverse conditions of gastrointestinal tract.

| **Isolates*** | **Time (min)** | **Conditions** | | | | |
|---|---|---|---|---|---|---|
| | | **Lysozyme** | **pH 2** | **pH 4** | **Bile salts 0.5%** | **Bile salts 1.0%** |
| BAL S34.1 | 180 | + | + | + | + | + |
| | 360 | + | + | + | + | + |
| BAL S34.10 | 180 | + | - | + | + | + |
| | 360 | + | - | + | + | + |
| BAL L56.1 | 180 | + | + | + | + | + |
| | 360 | + | + | + | + | + |
| BB A14.2 | 180 | + | + | + | + | + |
| | 360 | + | + | + | + | + |
| BB S34.5 | 180 | + | + | + | + | + |
| | 360 | + | + | + | + | + |
| BB R32.2 | 180 | + | + | + | + | + |
| | 360 | + | + | + | + | + |
| BL N49.3 | 180 | + | + | + | + | + |
| | 360 | + | + | + | + | + |
| BLL C25.3 | 180 | + | + | + | + | + |

| | | | | | | |
|---|---|---|---|---|---|---|
| | 360 | + | + | + | + | + |
| LB M24.2 | 180 | + | + | + | + | - |
| | 360 | + | + | + | + | - |
| LF L54.2 | 180 | + | + | + | + | + |
| | 360 | + | + | + | + | + |

The assays were performed with modified MRS with 300 μg/mL lysozyme, adjusted to pH 2 and pH 4, and supplemented with 0.5% and 1% bile salts, independently. Wells with non-adjusted MRS medium with and without inoculated bacteria were used as positive and negative controls, respectively. * BAL S34.1: *B. animalis* subsp. *lactis* S34.1; BAL L56.1 = *B. animalis* subsp. *lactis* L56.1; BB A14.2 = *B. bifidum* A14.2; BB S34.5 = *B. bifidum* S34.5; BB R32.2 = *B. breve* R32.2; BL N49.3 = *B. longum* N49.3; BLL C25.3 = *B. longum* subsp. *longum* C25.3; LB M24.2 = *L. brevis* M24.2; LF L54.2 = *L. fermentum* L54.2. The assays were performed in triplicate. The symbol + indicates survival ≥ 90% compared to positive control and – indicates survival < 90% compared to positive control.

**Table 4**
Antimicrobial activity assay of *Bifidobacterium* and *Lactobacillus* spp. isolated from children faeces against enteropathogens

| **Enteropathogens*** | **Inhibition halos of isolates in mm (± SD)**** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | BAL S34.1 | BAL L56.1 | BBF A14.2 | BBF S34.5 | BB R32.2 | BL N49.3 | BLL C25.3 | LF L54.2 | LF23271 | BLL15707 |
| EAEC | 10±0,2 | 10 | 15 | 9±0,1 | 12±0,8 | 19,5± 0,7 | 12±0,6 | 13±0,2 | 19 | 16,5±0,7 |
| EHEC | 12±1,8 | 12 | 11±0,6 | 11±1,6 | 10±0,9 | 17 | 16±0,8 | 16±0,2 | 15 | 18,5±2,8 |
| EPEC | 9±2,2 | 15 | 13±2,2 | 13±3,4 | 11±1,6 | 17 | 14±0,7 | 25 | 21 | 17,5±2,1 |
| ETEC | 14 | 11±0,8 | 14±2,8 | 12±0,8 | 14 | 12±0,2 | 14 | 15 | 13 | 18,6±1,54 |
| *S.* Choleraesuis | 14±2,8 | 13±3,2 | 17 | 12±0,6 | 13,5±2,8 | 11±0,2 | 14,5±2,1 | 13 | 12 | 22±1,4 |
| *S. flexneri* | 13 | 14 | 19±1,4 | 14±0,4 | 9±0,5 | 19,5± 0,7 | 15 | 10±0,4 | 20 | 20,5±0,7 |

*EAEC = enteroaggregative *E. coli* 042, EHEC = enterohemorrhagic *E. coli* EDL933, EPEC = enteropathogenic *E. coli* E2348/69, ETEC = enterotoxigenic *E. coli* 1661-1 LT/ST +. ** BAL S34.1: *B. animalis* subsp. *lactis* S34.1; BAL L56.1 = *B. animalis* subsp. *lactis* L56.1; BBF A14.2 = *B. bifidum* A14.2; BBF S34.5 = *B. bifidum* S34.5; BB R32.2 = *B. breve* R32.2; BL N49.3 = *B. longum* N49.3; BLL C25.3 = *B. longum* subsp. *longum* C25.3; LF L54.2 = *L. fermentum* L54.2; LF23271 = *L. fermentum* ATCC 23271; BLL15707 = *B. longum* subsp. *longum* ATCC 15707. All assays were performed in triplicate.

## 5 CONSIDERAÇÕES FINAIS

A seleção de novas linhagens de *Bifidobacterium* e de *Lactobacillus* com potencial probiótico e efetivas contra bactérias enteropatogênicas apresenta relevância em termos de saúde pública, principalmente quando consideramos os impactos negativos dos elevados índices de morbidade e de mortalidade por doença diarreica no âmbito mundial. Dentro desse contexto, apesar do painel de probióticos existentes no mercado, sabe-se que, na maioria dos casos, a ação benéfica desses microrganismos pode ser espécie-específica e, até mesmo, linhagem-específica. Portanto, certos produtos comercializados como probióticos não terão os mesmos efeitos nas diferentes desordens infeciosas e inflamatórias do trato intestinal, trato genital feminino e outras enfermidades, incluindo alergias, diabetes, entre tantas outras descritas na literatura.

À partir do desenvolvimento dessa tese foram obtidos os seguintes resultados:

- Produção do um artigo de revisão intitulado "*Probiotics, mechanisms of action, and clinical perspectives for diarrhea management in children*" que analisou o conhecimento atual sobre os mecanismos de ação dos probióticos frente aos enteropatógenos intestinais. Foi possível evidenciar, após a análise da literatura existente, a efetividade da aplicação clínica dos principais probióticos na prevenção e tratamento da diarreia infantil. Adicionalmente, foram discutidas algumas lacunas no conhecimento e desafios existentes, bem como as perspectivas dentro do ramo de pesquisa;
- Produção de um artigo experimental na modalidade *full paper* intitulado "Antimicrobial activity of *Bifidobacterium* and *Lactobacillus* species from children´s feces against bacterial enteropathogens" que demonstrou que os isolados *B. bifidum* A14.2, *B. longum* subsp. *longum* C25.3, *B. longum* N49.3, *B. animalis* subsp. *lactis* L56.1 e *L. fermentum* L54.2 apresentaram perfil adequado para serem considerados potenciais probióticos, além de inibirem enteropatógenos por meio da produção de compostos com atividade antimicrobiana e via competição por receptores de mucina. Tais achados são de fundamental importância para a ampliação do banco de microrganismos de origem brasileira com propriedades probióticas e que podem ser utilizados como alternativa preventiva ou terapêutica da doença diarreica.

## 6 CONCLUSÕES

Com base nos resultados deste trabalho foi possível concluir que:

- A partir da revisão sistemática de literatura, as cepas *Lactobacillus rhamnosus* GG, *Saccharomyces boulardii*, *L. acidophilus* e *L. reuteri* são capazes de reduzir o tempo de duração da diarreia infantil e de estadia hospitalar em, pelo menos, 24 horas. Além disso, a literatura descreve que uma alternativa interessante seria o *design* de probióticos a partir da microbiota endógena dos indivíduos. Isso poderia facilitar a eficácia desses compostos para a restauração/manutenção da homeostase da microbiota intestinal considerando-se as variações geográficas do microbioma dos indivíduos;
- As linhagens de *B. bifidum* A14.2, *B. longum* subsp. *longum* C25.3, *B. longum* N49.3, *B. animalis* subsp. *lactis* L56.1 e *L. fermentum* L54.2 apresentam propriedades benéficas considerando que apresentam o perfil para serem definidas como probióticos e atividade antimicrobiana contra bactérias enteropatogênicas. No entanto, existe a necessidade de estudos para confirmar a efetividade dessas bactérias em modelos animais de infecção experimental, para que se possa estabelecer a perspectiva de uso no tratamento da doença diarreica.

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# ANEXOS

## ANEXO A – PARECER CONSUBSTANCIADO DO CEP

CENTRO UNIVERSITÁRIO DO MARANHÃO - UNICEUMA

### PARECER CONSUBSTANCIADO DO CEP

**DADOS DO PROJETO DE PESQUISA**

**Título da Pesquisa:** IDENTIFICAÇÃO DE NOVOS MICRORGANISMOS COM POTENCIAL PROBIÓTICO CONTRA ENTEROPATÓGENOS BACTERIANOS

**Pesquisador:** VALÉRIO MONTEIRO NETO

**Área Temática:**

**Versão:** 1

**CAAE:** 35354614.5.0000.5084

**Instituição Proponente:** Centro Universitário do Maranhão - UniCEUMA

**Patrocinador Principal:** FUND DE AMPARO A PESQUISA AO DESEN CIENTIFICO E TECNOLOGICO DO MARANHÃO - FAPEMA

**DADOS DO PARECER**

**Número do Parecer:** 791.457

**Data da Relatoria:** 30/09/2014

**Apresentação do Projeto:**

O desenho do estudo visará obter novas espécies do gênero Lactobacillus e Bifidobacterium com potencial probiótico, oriundas de amostras de leite humano e fezes de lactentes e com atividade contra enteropatógenos bacterianos e/ou imunomoduladora. No estudo o delineamento experimento obedecerá o seguinte os seguintes passos: Isolar e identificar bactérias do gênero Lactobacillus e de Bifidobacterium de amostras de fezes de lactentes e de leite humano em diferentes estágios da lactação (colostro, leite de transição e leite maduro) de mães que tiveram parto prematuro e a termo; Selecionar as espécies com propriedades probióticas in vitro, incluindo: tolerância ao suco gástrico artificial e aos sais biliares; capacidade antagonista contra patógenos intestinais, capacidade de produção de peróxido de hidrogênio; capacidade de adesão em células eucarióticas intestinais in vitro e à mucina; Investigar a capacidade inibitória sobre a adesão de enteropatógenos bacterianos em células eucarióticas in vitro; Verificar a capacidade de indução de citocinas pró e anti-inflamatórias em células de cultura de tecidos; Avaliar a capacidade de produção de peróxido de hidrogênio e nitritos por células apresentadoras de antígenos; Investigar o espraiamento de células apresentadoras de antígenos; Estudar a indução de marcadores de ativação e estado funcional de células dendríticas, macrófagos e linfócitos in vitro e ex vivo; Analisar a expressão de receptores TLR em leucócitos de camundongos

**Endereço:** DOS CASTANHEIROS
**Bairro:** JARDIM RENASCENCA **CEP:** 65.075-120
**UF:** MA **Município:** SAO LUIS
**Telefone:** (98)3214-4265 **Fax:** (98)3214-4212 **E-mail:** cep@ceuma.br

estimulados com Probióticos. As amostras de leite serão coletas de lactentes que apresentarem as características necessárias definidas no escopo do trabalho. Serão realizadas coletas em cada estágio de lactação (colostro, leite de transição e leite maduro) de mães que tiveram parto a termo (37 e < 42 semanas) e prematuro com idade gestacional ( 34 e < 37 semanas), cadastradas no Banco de Leite do Hospital Materno Infantil do Hospital Universitário da UFMA. As amostras de fezes serão obtidas de crianças sem distúrbios gastrointestinais e que não estejam sob uso de antibióticos, cujos pais ou responsáveis aceitarem participar do estudo.

**Objetivo da Pesquisa:**

Identificar novas espécies do gênero Lactobacillus e Bifidobacterium com potencial probiótico, oriundas de amostras de leite humano e fezes de lactentes e com atividade contra enteropatógenos bacterianos e/ou imunomoduladora.

**Avaliação dos Riscos e Benefícios:**

Riscos:

A participação nesta pesquisa não traz complicações legais. Somente haverá um pequeno desconforto quanto a retirada de uma amostra leite, mas tudo na maior segurança e com profissionais qualificados. A coleta das fezes ocorrerá diretamente nas fraldas e não oferece riscos e nem desconforto. Os procedimentos adotados nesta pesquisa obedecem aos Critérios da Ética em Pesquisa com Seres Humanos conforme Resolução

no. 466/12 do Conselho Nacional de Saúde. Nenhum dos procedimentos usados oferece riscos à sua dignidade.

Benefícios:

Espera-se que este estudo traga informações importantes sobre os benefícios dessas bactérias para a nossa saúde, principalmente de crianças.

**Comentários e Considerações sobre a Pesquisa:**

A pesquisa apresenta relevância científica e certamente contribuirá para esclarecer aspectos importantes a respeito do tema. A equipe executora apresenta a capacitação necessária para realizar a pesquisa.

**Considerações sobre os Termos de apresentação obrigatória:**

Todos os documentos obrigatórios foram apresentados e encontram-se corretamente preenchidos.

CENTRO UNIVERSITÁRIO DO MARANHÃO - UNICEUMA

Continuação do Parecer: 791.457

**Recomendações:**

Nenhuma

**Conclusões ou Pendências e Lista de Inadequações:**

Projeto aprovado

**Situação do Parecer:**

Aprovado

**Necessita Apreciação da CONEP:**

Não

**Considerações Finais a critério do CEP:**

SAO LUIS, 15 de Setembro de 2014

**Assinado por:**
**Eduardo Durans Figuerêdo**
**(Coordenador)**

ANEXO B – DECLARAÇÃO DA SECRETARIA MUNICIPAL DE SAÚDE

Prefeitura e você, construindo um novo caminho
SECRETARIA DE SAÚDE / SEMUS

SUPERINTENDÊNCIA DE REDE DE ASSISTÊNCIA A SAÚDE

# DECLARAÇÃO

Declaro para os devidos fins, que o **aluno (a)** Monique Santos do Carmo **do (a)** Doutorado em Ciências da Saúde - UFMA, está autorizado (a) a coletar dados, para a realização do Projeto: Influência da microbiota intestinal na expressão de fatores de adesão de Escherichia coli diarreiogênicas, na Unidade Dr. Odorico Amaral de Matos de nossa Rede de Saúde, **após a aprovação do referido Projeto por um Comitê de Ética em Pesquisa.**

São Luís, 19 de fevereiro de 2015.

CEP 791.457

Atenciosamente,

Lídia Saldanha Nicolau
MÉDICA - CRM 1719
CPF: 129.378.513-04

**Lídia Saldanha Nicolau**
**Superintendente de Educação em Saúde / SEMUS**

APÊNDICE A - TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

UNIVERSIDADE CEUMA

**Comitê de Ética em Pesquisa**

**Título da Pesquisa:** "**PROSPECÇÃO DE MICRORGANISMOS COM POTENCIAL PROBIÓTICO CONTRA ENTEROPATÓGENOS BACTERIANOS**"

**Nome do Pesquisador:** Valério Monteiro Neto

1. **Natureza da pesquisa**: *A Sra. está sendo convidada a participar desta pesquisa que tem como finalidade analisar bactérias presentes em fezes de crianças, em condições normais e com isso verificar os seus benefícios para a saúde humana e propor medidas alternativas de controle e prevenção de determinadas doenças, como a diarreia infantil.*
2. **Participantes da pesquisa***: Serão incluídas crianças com a faixa etária entre 1 mês e cinco anos de idade.*
3. **Envolvimento na pesquisa**: *a sua participação no referido estudo será no sentido de fornecer uma pequena quantidade de fezes do seu filho (a).*
4. *A Sra tem liberdade de se recusar a participar e ainda se recusar a continuar participando em qualquer fase da pesquisa, sem qualquer prejuízo para o seu filho. Sempre que quiser poderá pedir mais informações sobre a pesquisa através do telefone do pesquisador do projeto e, se necessário, através do telefone do Comitê de Ética em Pesquisa.*
5. **Sobre as entrevistas**: *As entrevistas serão realizadas no Hospital Odorico Amaral de Matos – "Hospital da Criança"* .
6. **Riscos e desconforto**: *A participação nesta pesquisa não traz complicações legais. A coleta das fezes ocorrerá diretamente nas fraldas e não oferece riscos e nem desconforto. Os procedimentos adotados nesta pesquisa obedecem aos Critérios da Ética em Pesquisa com Seres Humanos conforme Resolução no. 466/12 do Conselho Nacional de Saúde. Nenhum dos procedimentos usados oferece riscos à sua dignidade*.
7. **Confidencialidade**: *Todas as informações coletadas neste estudo são estritamente confidenciais. Somente os pesquisadores terão conhecimento dos dados*.

8. **Benefícios**: *Ao participar desta pesquisa a Sra. não terá nenhum benefício direto. Entretanto, esperamos que este estudo traga informações importantes sobre os benefícios dessas bactérias para a nossa saúde, principalmente de crianças.*
9. **Pagamento**: *A Sra não terá nenhum tipo de despesa para participar desta pesquisa, bem como nada será pago por sua participação*.

Após estes esclarecimentos, solicitamos o seu consentimento de forma livre para participar desta pesquisa. Portanto preencha, por favor, os itens que se seguem:

Consentimento Livre e Esclarecido

Tendo em vista os itens acima apresentados, eu, de forma livre e esclarecida, manifesto meu consentimento em participar da pesquisa.

_____ _____

Nome do Participante da Pesquisa Assinatura do participante da pesquisa

_____

Assinatura do Pesquisador

**TELEFONES**

**Pesquisador:** Prof. Dr. Valério Monteiro Neto (98) 9972-2651 e 3214 4252

**Comitê de Ética em Pesquisa do Uniceuma:**
Endereço: Rua Josué Montello, nº1, Renascença II, São Luís-MA, CEP 65.075-120.

Telefone: (98) 3214-4189. E-mail: cep@ceuma.br.

APÊNDICE B – FICHA DE IDENTIFICAÇÃO DO PACIENTE

Universidade Federal do Maranhão
Centro de Ciências Biológicas e da Saúde
Programa de Pós-Graduação em Ciências da Saúde – Doutorado
Doutoranda: Monique Santos do Carmo

**Ficha de identificação do paciente**

**Título da pesquisa: "PROSPECÇÃO DE MICRORGANISMOS COM POTENCIAL PROBIÓTICO CONTRA ENTEROPATÓGENOS BACTERIANOS"**

**1- Identificação da criança:**

1.1 - Nome completo:

1.2 - Idade:

1.3 - Está sob o uso de antibióticos? Em caso negativo, há quanto tempo?

( )Sim ( )Não Período: _____

1.4 – A criança tem apresentado três ou mais evacuações líquidas ou de fezes amolecidas no período de 24 horas, há menos de sete dias?

( )Sim ( )Não

1.5- Qual tipo de parto?

( )cesárea ( )normal

1.6- Tipo de nascimento:

( ) à termo ( )pré-termo

**2- Identificação da mãe:**

2.1 – Nome completo:

2.2 – Idade:

2.3 – Endereço:

Ponto de referência:

2.4 – Telefone: