Universidade Federal do Maranhão
Centro de Ciências Biológicas e da Saúde
Programa de Pós-Graduação em Ciências da Saúde
Mestrado em Ciências da Saúde

# **Atividade antimicrobiana *in vitro* e potencial probiótico de *Bifidobacterium e Lactobacillus* contra espécies de *Clostridium***

CINARA REGINA ARAGÃO VIEIRA MONTEIRO

São Luís
2018

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências da Saúde da Universidade Federal do Maranhão – UFMA, como requisito para obtenção do título de Mestre em Ciências da Saúde.

Orientador: Prof.º Dr. Valério Monteiro Neto

Co-orientadora: Prof.ª Dra. Maria Rosa Quaresma Bonfim

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Aprovada em / /

BANCA EXAMINADORA

_____

Prof.º Dr. Valério Monteiro Neto (Orientador)

Universidade Federal do Maranhão

_____

Prof.º Dr. Afonso Gomes Abreu Junior

Universidade Federal do Maranhão

_____

Prof.ª Dra. Cristina de Andrade Monteiro

Universidade Ceuma

_____

Prof.ª Dra. Lucilene Amorim Silva

Universidade Federal do Maranhão

“A única maneira de fazer um excelente trabalho é amar o que você faz. Se você ainda não encontrou, continue procurando. Não se acomode. Assim como tudo que importa para o coração, você saberá quando encontrar. ”

*Steve Jobs*

Dedico

Aos meus amores Rodrigo e
Giovanna

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# SUMÁRIO

# LISTA DE SIGLAS E ABREVIATURAS

| | |
|---|---|
| **ATCC** | American Type Culture Collection |
| **CPE** | *Clostridium perfringens* enterotoxin |
| **DNA** | Ácido Desoxiribonucléico |
| **EN** | Enterocolite Necrosante |
| **IgA** | Imunoglobulina tipo A |
| **INCQS** | Instituto Nacional de Controle de Qualidade em Saúde |
| **MRS** | Man-Rogosa-Stuart |
| **NCIMB** | National Collection of Industrial, Food and Marine Bacteria |
| **PMC** | Colite Pseudomembranosa |
| **RCM** | Reinforced Clostridial Medium |
| **TcdA** | Toxina *Clostridium Difficile* tipo A |
| **TcdB** | Toxina *Clostridium Difficile* tipo B |
| **TGI** | Trato Gastrointestinal |
| **UFC** | Unidades Formadoras de Colônias |
| **DII** | Doença Inflamatória Intestinal |
| **DAA** | Diarreia Associada a Antibiótico |
| **$H_2$** | Hidrogênio |
| **$CO_2$** | Dióxido de Oxigênio |

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## RESUMO

Espécies de *Clostridium*, principalmente *C. butyricum*, *C. difficile* e *C. perfringens*, são agentes de doenças infecciosas resultantes de disbiose intestinal. Em muitos casos, a recorrência pode ocorrer após antibioticoterapia, indicando a necessidade de novas opções terapêuticas que atuem sobre os patógenos e também restaurem a microbiota. Neste trabalho, investigou-se a atividade antimicrobiana *in vitro* e o potencial probiótico de cepas clínicas e de referência de *Bifidobacterium* e *Lactobacillus* contra espécies patogênicas de *Clostridium*. A atividade antimicrobiana foi avaliada pelo ensaio de difusão em sobrecamadas e pela inibição da produção de gás. Em seguida, o potencial probiótico das cepas selecionadas foi avaliado pela análise da capacidade de coagregação, propriedades de adesão às células eucarióticas e mucina, tolerância ao pH ácido e sais biliares, e pela determinação do perfil de susceptibilidade aos antibióticos. *Lactobacillus plantarum* ATCC 8014 foi a cepa mais promissora baseada em sua atividade inibitória contra *Clostridium* spp. Além disso, esta cepa preencheu os critérios para ser considerada um probiótico com base na sua capacidade de coagregação, propriedades de adesão e tolerância a condições adversas de pH e sais de ácidos biliares. No geral, os resultados indicam que, entre as linhagens estudadas, *L. plantarum* ATCC 8014 apresenta potencial probiótico para o controle de infecções pelas espécies de *Clostridium* estudadas e deve ser melhor avaliado em estudos animais.



Palavras-chave: Probióticos; *Lactobacillus plantarum*; *Clostridium*; Disbiose; Atividade antimicrobiana.

## ABSTRACT

*Clostridium* species, mainly *C. butyricum*, *C. difficile*, and *C. perfringens,* are agents of infectious disease resulting from intestinal dysbiosis. In many cases, recurrence can occur after antibiotics, indicating the need for novel therapeutic options that act on the pathogens and also restore the microbiota. Herein, the *in vitro* antimicrobial activity and probiotic potential of clinical and reference strains of *Bifidobacterium* and *Lactobacillus* were investigated against pathogenic species of *Clostridium*. The antimicrobial activity was evaluated by the agar spot test and inhibition of gas production. Then, the probiotic potential of selected strains was assessed by analyzing their coaggregation ability, adhesive properties to host cells and mucin, tolerance to acidic pH and bile salts, and antimicrobial susceptibility profiles. *Lactobacillus plantarum* ATCC 8014 was the most promising strain based on its inhibitory activity against *Clostridium* spp. In addition, this strain met criteria to be considered a probiotic based on its coaggregation ability, adhesive properties, and tolerance to harsh conditions of pH and bile acid salts. Overall, the results indicate that among the studied strains, *L. plantarum* ATCC 8014 presents probiotic potential for controlling infections by the *Clostridium* species studied and should be further evaluated in *in vivo* animal studies.

**Keywords:** probiotics; *Lactobacillus plantarum*; *Clostridium*; dysbiosis; antimicrobial activity

# 1. INTRODUÇÃO

A microbiota de determinados sítios anatômicos do corpo humano, tais como a pele, trato respiratório superior, cavidade oral, trato gênito-urinário e trato gastrointestinal, apresentam uma grande diversidade de microrganismos que desempenham papéis importantes à saúde e ao metabolismo do ser humano. Estima-se que o número total de microrganismos da microbiota humana seja tão elevado que supera o número de células eucarióticas do organismo. Desses sítios anatômicos, a microbiota intestinal é incomparavelmente a que representa a maior diversidade de filos ou taxos, particularmente no cólon (KAMADA et al., 2013).

Adicionando ainda mais a essa complexidade, há também um elevado grau de variação interindividual no conteúdo de espécies bacterianas, o que significa que não há espécies microbianas "básicas" que estejam presentes em todos os seres humanos. Embora exista um grande grau de variação no conteúdo de espécies de microbiota intestinal entre indivíduos, há uma série de características consistentes que caracterizam a microbiota na saúde, a composição das espécies dentro dos hospedeiros individuais parece ser bastante estável durante períodos de tempo prolongados, mas a abundância relativa de algumas espécies constituintes parece variar constantemente em resposta a fatores como mudanças na dieta do hospedeiro (JIMÉNEZ et al., 2008).

Outra característica predominante da microbiota associada à saúde é que as bactérias anaeróbias obrigatórias superam em grande parte as espécies anaeróbias facultativas. De um modo geral, a grande maioria da microbiota intestinal pertence aos filos dos Bacteroidetes e Firmicutes, embora os membros do filo de Actinobacterias, como Bifidobactérias e Proteobactérias também possam estar relacionados (DONALDSON et al., 2015).

A microbiota intestinal é descrita como altamente benéfica à saúde do hospedeiro. Por outro lado, ela é também a fonte de compostos potencialmente danosos e de patógenos oportunistas. Desta forma, a saúde do hospedeiro está intimamente ligada ao seu equilíbrio balanceado. Em contraste, o termo "disbiose" refere-se a situações em que a composição microbiana e as atividades são deslocadas do seu estado normal e benéfico para outro que é mais deletério para a saúde do hospedeiro. De fato, numerosos distúrbios, tanto intestinais quanto sistêmicos, tem sido ligados à disbiose intestinal, incluindo-se diarreia associada a antibióticos, enterocolite necrosante, colite pseudomembranosa, doença de Crohn, entre outras

(CHAMBRUN et al., 2008; KAUR et al., 2011; PROSBERG et al., 2016; McFARLAND et al., 2016; PAMMI et al., 2017).

Entretanto, ainda não há uma clara compreensão de todos os aspectos etiológicos envolvidos na patogênese dessas doenças, existem evidências que apontam para o envolvimento de algumas espécies de *Clostridium*, entre outros patógenos oportunistas. Das espécies comumente relatadas, destacam-se *Clostridium butyricum*, *Clostridium difficile* e *Clostridium perfingens* (SOMMER; BACKHED, 2013; CASSIR et al., 2016).

Dado os papéis potencialmente importantes que a microbiota pode desempenhar na manutenção da homeostase ou na etiologia de várias doenças, a manipulação terapêutica da comunidade microbiana intestinal é um objetivo desejável. De um modo geral, isso pode ser alcançado com várias abordagens diferentes, como pela(o): 1) estimulação de respostas imunes do hospedeiro, 2) uso de drogas que atenuam especificamente atividades deletérias ou metabólitos da microbiota, 3) eliminação de espécies bacterianas através de antibióticos e 4) inoculação de espécies benéficas. Esta última estratégia é obtida pela ingestão de microrganismos designados de probióticos e aparenta ser a mais racional, considerando que a pressão seletiva a ser exercida afetaria menos o conjunto de bactérias que compõem a microbiota. As principais bactérias ditas como probióticas pertencem aos gêneros *Bifidobacterium* e *Lactobacillus* (EMAMI et al., 2009; TLASKALOVA-HOGENOVA et al., 2011; HILL et al., 2014; KUMAR; KUMAR, 2015).

As evidências atuais quanto a eficácia dos probióticos é variada, porém, é confundida pelo fato de que os regimes de tratamento em diferentes estudos são altamente heterogêneos, com diferentes espécies ou linhagens, dosagens, durações de tratamento e métodos de administração empregados. Além disso, dados clínicos recentes sugerem que probióticos específicos podem ser opções efetivas de tratamento para distúrbios relacionados a determinados casos de disbiose intestinal, tais como na prevenção de enterocolite necrosante em lactentes e na prevenção de diarreia associada aos antibióticos.

Apesar das evidências em pacientes, ainda não há comprovação *in vitro* da atividade antagônica contra os patógenos frequentemente envolvidos nessas síndromes clínicas. Portanto, este estudo teve por objetivo realizar seleção *in vitro* de linhagens de referência e de isolados clínicos de espécies de *Bifidobacterium* e *Lactoctobacillus* com atividade inibitória do crescimento de *Clostridium butyricum*, *Clostridium difficile* e *Clostridium perfringens*.

# 2. REFERENCIAL TEÓRICO

## 2.1 Desenvolvimento da microbiota intestinal

A microbiota intestinal desempenha papel importante na vida humana, funcionando como barreira epitelial protetora do contra microrganismos patogênicos, auxiliando nas reações metabólicas, modulação de respostas imunes inatas e adaptativas do hospedeiro (SEKIROV; FINLAY, 2009; LITTMAN; PAMER, 2011; MATAMOROS et al., 2013; PEREZ-LOPEZ et al., 2016).

O desenvolvimento da microbiota inicia-se desde a vida intrauterina evoluindo seu amadurecimento e atingindo o estado adulto por volta dos 3 anos de idade, durante esse período é influenciado pela microbiota de adultos que convivem no mesmo ambiente familiar (PALMER et al., 2007; MATAMOROS et al., 2013).

Acreditava-se que o ambiente intrauterino e o recém-nascido eram estéreis até o parto, porém estudos do líquido amniótico e placenta maternos (DIGIULIO et al., 2008; SATOKARI et al., 2009; COLLADO et al., 2016) e do mecônio infantil (MADAN et al., 2012; COLLADO et al., 2016) revelaram a presença de microrganismos, sugerindo transferência microbiana. Considera-se assim que a via hematogênica corresponde a principal interface para translocação de bactérias intestinais da mãe para o feto (JIMÉNEZ et al., 2008).

Fatores externos à gravidez também podem influenciar no desenvolvimento da microbiota, tais como: uso de drogas, doenças, estresse, exposição a metais pesados, dieta e tratamentos com probióticos e antibióticos durante a gestação (BAILEY et al., 2004; LAHTINEN et al., 2009; MATAMOROS et al., 2013).

O tipo de parto apresenta influência direta na colonização intestinal inicial. Estudos demonstram que neonatos nascidos de parto vaginal sofrem maior influência da microbiota da genitália feminina e apresentam maior diversidade bacteriana quando comparadas com crianças nascidas de parto cesariano que sofrem maior influência da pele materna (DOMINGUEZ-BELLO et al., 2010; RUTAYISIRE et al., 2016).

O leite materno proporciona uma variedade de bactérias comensais e mutualísticas, com predomínio de *Staphylococcus spp*, *Streptococcus spp*, bactérias do ácido lático e

*Bifidobacterium spp.* A microbiota intestinal materna atinge as glândulas mamárias e colonizam o lactente durante o período de lactação, contribuindo também na maturação das respostas imunológicas (FERNÁNDEZ et al., 2012). Os principais fatores que influenciam no desenvolvimento da microbiota estão resumidos na Figura 1.

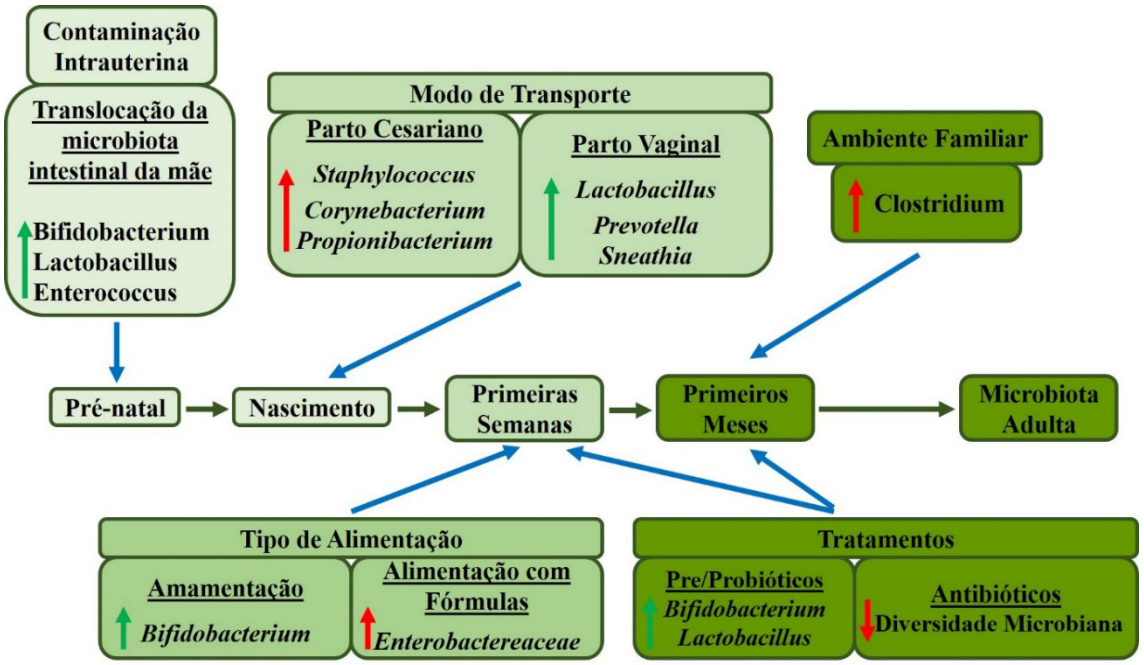


**Figura 1:** Fatores associados ao desenvolvimento da microbiota intestinal infantil (Fonte: MATAMOROS et al., 2013; modificado).

Inicialmente, a microbiota intestinal infantil é predominantemente colonizada por bactérias anaeróbias facultativas, como *Escherichia coli* e outros membros da família Enterobacteriaceae (JIMÉNEZ et al., 2008). Com a redução do suprimento de oxigênio por essas bactérias, o intestino torna-se um ambiente propício para o crescimento de microrganismos anaeróbios estritos como *Bacteroides spp, Bifidobacterium spp* e *Clostridium spp*. Essa baixa diversidade e complexidade bacterianas que caracterizam essa fase altera-se gradualmente até aproximadamente os 3 anos (MATAMOROS et al., 2013). Desses gêneros, as espécies de *Bifidobacterium* são os microrganismos dominantes na microbiota nesse período (TURRONI et al., 2012).

A composição biogeográfica na microbiota intestinal compreende as regiões específicas dos trato gastrointestinal (TGI) no qual as características químicas e fisiológicas

interferem diretamente na formação microbiana em diferentes locais, definindo, desta forma, a microbiota específica para cada região (Figura 2) (DONALDSON et al., 2015).

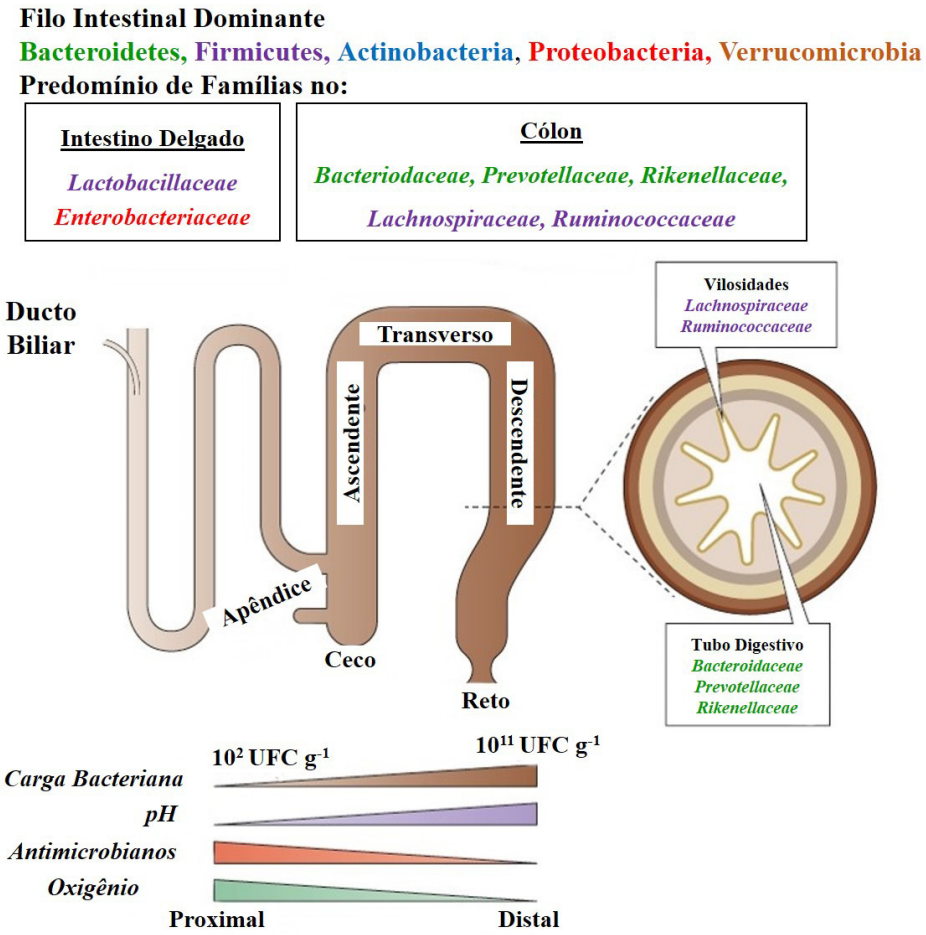


**Figura 2:** Composição da microbiota intestinal por região do trato gastrointestinal (Fonte: DONALDSON et al., 2015; modificado).

## 2.2 Probióticos e equilíbrio do ecossistema intestinal

Entre os microrganismos que compõem a microbiota intestinal existem espécies que atuam em uma relação de comensalismo, sem promover um benefício direto ao hospedeiro. Contudo, outros exercem ação mutualística, influenciando positivamente no metabolismo, na nutrição, no sistema imune e, até mesmo, apresentando atividade antimicrobiana. Esse aspecto tem sido evidenciado em estudos *in vitro* e *in vivo* que demonstraram que o ecossistema

bacteriano intestinal normal é extremamente eficaz em oposição a microrganismos patogênicos e oportunistas (EMAMI et al., 2009; TLASKALOVA-HOGENOVA et al., 2011).

Os microrganismos que exercem ações positivas à saúde do ser humano têm sido designados de probióticos. Segundo a Organização das Nações Unidas para Agricultura e Alimentação e a Organização Mundial de Saúde, probióticos são microrganismos vivos que, quando administrados em quantidades adequadas, conferem benefícios à saúde do hospedeiro (FAO/WHO, 2001). Os benefícios alcançados pelos probióticos possuem ação abrangente (Figura 3) (HILL et al., 2014). Assim, destacam-se a diminuição da diarreia em um menor tempo e consequentemente do tempo de hospitalização (VANDENPLAS, 2016), melhora da resposta imune nos diversos quadros alérgicos (DEL GIUDICE et al., 2017; DENNIS-WALL et al., 2017), prevenção do câncer do TGI (KAHOULI et al., 2017; SABER et al., 2017) e no controle dos níveis glicêmicos em pacientes diabéticos (HU et al., 2017; WANG et al., 2017).

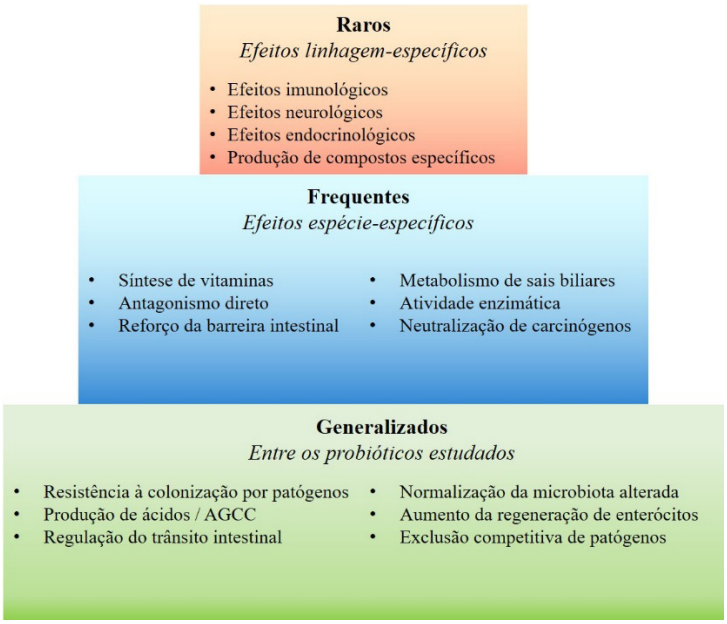


**Figura 3:** Benefícios dos probióticos (Fonte: HILL et al., 2014, modificado).

Várias linhagens têm sido definidas como probióticos, principalmente algumas pertencentes às espécies dos gêneros *Bifidobacterium*, *Lactobacillus, Saccharomyces, Streptococcus*, *Escherichia*, *Lactococcus* e *Enterococcus*, sendo estes dois últimos em menor escala (DE VRESE, SCHREZENMEIR, 2008; HILL et al., 2014; KUMAR; KUMAR, 2015).

*Lactobacillus* são microrganismos anaeróbios facultativos ou estritos, Gram-positivos, catalase negativa, não formadores de esporos, encontrados na microbiota normal humana de diferentes sítios anatômicos, incluindo o intestino (ZOETENDAL et al., 2006; KUMAR; KUMAR, 2015). Enquanto que, *Bifidobacterium* são bactérias Gram-positivas e anaeróbias estritas que também estão presentes normalmente no TGI de seres humanos e de animais, e não formam esporos (REUTER, 2001; TURRONI et al., 2011).

Evidências oriundas de experimentos com modelos animais reforçam os efeitos benéficos dos probióticos para a saúde humana. Estudos com *Lactobacillus casei* demonstram uma redução na permeabilidade intestinal em um modelo animal de colite aguda (ZAKOSTELSKA et al., 2011). Em ensaios com ratos, tratados com *Bifidobacterium lactis,* foi observado que há melhora do peristaltismo dos animais em comparação ao grupo controle (MATSUMOTO et al., 2012). Além disso, a administração de *L. reuteri* é capaz de promover diminuição na expressão de marcadores pró-inflamatórios em ratos recém-nascidos com enterocolite necrosante (EN) (LIU et al., 2012).

Após a comprovação científica das propriedades benéficas ao hospedeiro, vários produtos passaram a oferecer a suplementação dessas estirpes em sua formulação, incluindo iogurtes, leite fermentado e não-fermentado, queijo, sucos, *smoothies*, barras de cereais, dentre outras fórmulas infantis (CHAMPAGNE et al., 2005). Adicionalmente, os probióticos são comercializados como suplementos dietéticos e medicamentos (FOLIGNÉ et al., 2013; PASSARIELLO et al., 2014) (Figura 4).

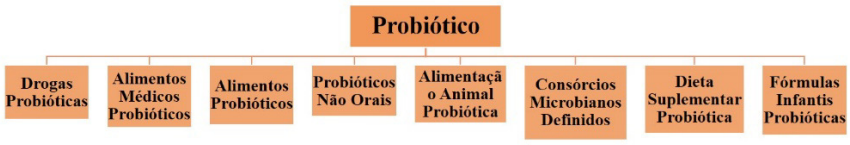


**Figura 4:** Formas de consumo probiótico (Fonte: HILL et al., 2014, modificado).

Portanto, é razoável supor que a administração de probióticos poderia ser uma alternativa válida para o controle de infecções do TGI em todas as fases de desenvolvimento, em particular nos casos decorrentes de desequilíbrio da microbiota, inclusive em neonatos prematuros. Principalmente porque o intestino prematuro é mais susceptível a inflamações e lesões devido à permeabilidade aumentada, diminuição do peristaltismo aumentando o risco de

translocação bacteriana, alteração na produção do muco e diminuição da secreção de IgA. O uso de probióticos, isolados ou em combinação, poderiam modificar tais alterações (FROST, CAPLAN, 2013).

## 2.3 *Clostridium* e disbiose intestinal

A estabilidade da homeostase intestinal pode ser influenciada por vários fatores que incluem o pH intestinal, a fisiologia do hospedeiro, as interações entre os microrganismos, uso de antibióticos, funcionamento intestinal e resposta do sistema imunológico. O desequilíbrio em algum desses fatores propiciam a colonização de patógenos, levando ao desenvolvimento da disbiose (Figura 5) (BÄCKHED et al., 2005; THOMPSON-CHAGOYÁN et al., 2007; CONLON; BIRD, 2014).

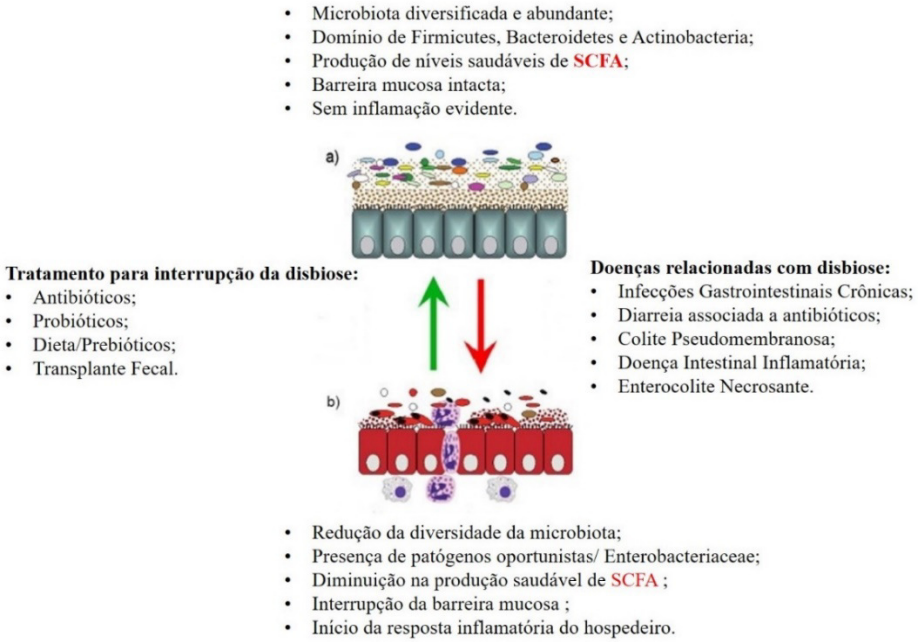


**Figura 5:** Correlação entre disbiose e homeostase intestinal (Fonte: WALKER; LAWLEY, 2013; modificado).

O termo disbiose intestinal, que surgiu da teoria da toxemia intestinal, refere-se a mudanças qualitativas e quantitativas da microbiota intestinal, incluindo distribuição bacteriana local e atividade metabólica. A dieta, estilo de vida e o uso de antibióticos podem alterar a

microbiota intestinal normal, tornando o ambiente propício à colonização e proliferação de enteropatógenos potenciais, os quais produzem metabólitos tóxicos (HAWRELAK; MYERS, 2004).

Um desequilíbrio na microbiota intestinal pode causar ou contribuir para o estabelecimento de doenças infecciosas e inflamatórias, como doença inflamatória intestinal (DII) (CHAMBRUN et al., 2008; KAUR et al., 2011; PROSBERG et al., 2016), diarreia associada a antibióticos (DAA) (McFARLAND et al., 2016), síndrome do intestino irritável (BENNET et al., 2015) e enterocolite necrosante (EN) (PAMMI et al., 2017). Além disso, há crescentes evidências revisadas na literatura sobre a associação de disbiose com outras doenças de natureza não-infecciosa, incluindo diabetes tipo 2 (SIRCANA et al., 2018), asma (DURACK et al., 2016), doença hepática gordurosa não alcoólica (BOURSIER et al., 2016), câncer colorretal (TILG et al., 2018), condições neurológicas (TREMLETT et al., 2017) e doenças cardiovasculares (LEZUTEKONG et al., 2018).

O tratamento da disbiose têm evoluído ao longo dos tempos e, geralmente, é baseada em: 1) antibioticoterapia, 2) transplante ou bacterioterapia fecal e 3) uso de prebióticos e probióticos, além da adequação da dieta. Contudo, o nível considerável da resistência bacteriana aos antibióticos tem feito com que a terapia seja cada vez mais prolongada e por uso de múltiplos antibióticos, o que altera ainda mais a microbiota. Por outro lado, a bacterioterapia fecal, ou seja, a substituição da microbiota intestinal alterada a partir de um doador saudável, tem resultado em boa evolução clínica dos pacientes, porém estudos são necessários para elucidação dos mecanismos de ação envolvidos, do perfil microbiano adequado, bem como da resposta imune do hospedeiro para o controle do processo infeccioso (KELLY et al., 2012; WALKER; LAWLEY, 2013).

Dentro desse contexto, o uso de probióticos têm demonstrado ser uma conduta mais racional, associada ou não à terapia com antibióticos, para a abordagem dos pacientes com disbiose intestinal; contribuindo para uma microbiota saudável (WALKER; LAWLEY, 2013; HILL et al., 2014). Além do que, não haveria a mesma preão eletiva sobre as bactérias da microbiota, exercida pela antibioticoterapia, proporcionando mais desequilíbrio no ecossistema intestinal.

Entre as bactérias do filo Firmicutes está o gênero *Clostridium* que é constituído por bacilos Gram-positivos, anaeróbios estritos, capazes de formar esporos em situações

desfavoráveis para seu crescimento e metabolismo, podendo assim sobreviver por um tempo prolongado no organismo do hospedeiro ou no meio ambiente (CASSIR et al., 2016).

Espécies do gênero *Clostridium* são encontradas na microbiota comensal do intestino de animais e humanos, onde vivem como saprófitas, porém em determinadas situações que resultam em desequilíbrio da microbiota, algumas espécies multiplicam-se excessivamente e passam a exercer ação enteropatogênica com grande repercussão para a saúde humana, tais como: *Clostridium butyricum*, *Clostridium difficile* e *Clostridium perfringens* (SOMMER; BACKHED, 2013).

*C. butyricum* é um microrganismo que fermenta uma variedade de substratos, principalmente carboidratos, produzindo diferentes tipos de ácidos orgânicos de cadeia curta, bem como gás hidrogênio ($H_2$) e dióxido de carbono ($CO_2$), como resultado do seu processo metabólico (KAO et al., 2014). Existe controvérsia com relação a sua patogenicidade, pois algumas linhagens de *C. butyricum* são utilizadas como probiótico, enquanto outras estão implicadas em condições patológicas, tais como botulismo e enterocolite necrosante (EN) em neonatos prematuros. Os dados da literatura sugerem que a capacidade de causar doença está relacionada não somente à produção de toxinas histolíticas, enterotoxigênicas e neurotoxinas, como também pela habilidade de sobreviver em condições ambientais adversas pelo processo de esporulação e de multiplicação rápida (HOWARD et al., 1977, FENICIA et al., 2007; MOROWITZ et al., 2010, CASSIR et al., 2015; CASSIR et al., 2016).

A segunda espécie, *C. difficile*, também é encontrada no trato gastrointestinal (TGI), mas em um número mais reduzido de indivíduos. A bactéria possui disseminação pela via fecal-oral, principalmente pelas mãos da equipe multidisciplinar hospitalar e por outros pacientes infectados. A patogênese da infecção é caracterizada por doença gastrointestinal adquirida pós-antibioticoterapia, principalmente em pacientes internados. A síndrome clínica pode variar de uma diarreia autolimitada até uma colite pseudomembranosa (PMC) grave e letal, particularmente em crianças e idosos com hospitalização recente ou residentes em asilos (SURAWICZ et al., 2013; CREWS et al., 2014).

O tratamento com antibióticos altera a microbiota normal, favorecendo o crescimento desse microrganismo, o qual é altamente resistente a antimicrobianos e produtor de duas toxinas de natureza protéica: toxina A do *C. difficile* (TcdA) e toxina B do *C. difficile* (TcdB) (SANDORA et al., 2011; KIM et al., 2012; SAMMOS et al., 2013; ABT et al., 2016). Os flagelos existentes nesse patógeno proporciona a ligação com as células epiteliais intestinais, favorecendo assim a ação das exotoxinas que causam inflamação intestinal e diarreia (PRUITT;

LACY, 2012; STEVENSON et al., 2015; ABT et al., 2016; ANJUWON-FOSTER; TAMAYO, 2017).

A terceira espécie, *C. perfringens,* também está associada a uma desordem intestinal, apesar de poder ser encontrada normalmente no TGI de animais e humanos. Adicionalmente, possui como habitat natural o solo e pode contaminar alimentos, pois os esporos termorresistentes sobrevivem ao cozimento e germinam posteriormente. Possuem um crescimento rápido, formando colônias espalhadas e grande potencial patogênico devido à ação de enterotoxinas que atuam como superantígenos. Até o momento, 13 toxinas já foram descritas para a espécie, contudo as dos tipos A a E são consideradas letais (Tabela 1) (PETIT et al., 1999; BRYNESTAD; GRANUM, 2002; UZAL et al, 2014).

**Tabela 1:** Principais doenças associadas com *Clostridium perfringens* em animais e humanos (Fonte: UZAL et al., 2014, modificada)

| Tipos de *Clostridium perfringens* | Toxinas Produzidas | Doenças mais significantes |
|---|---|---|
| A | CPA | Gangrena Gasosa em humanos e animais |
| | CPA, CPE | Intoxicação alimentar e não alimentar |
| | CPA, NetB | Enterite Necrótica em animais |
| B | CPA, CPB, ETX | Enterite Necrohemorrágica em animais |
| C | CPA, CPB | Enterite Necrótica Humana, Enterite Necrótica Neonatal em animais |
| D | CPA, ETX | Enterotoxemia em animais |
| E | CPE, ITX | Doenças gastrointestinal em animais |

*CPA = Alpha; CPB = Beta; ETX = Epsilon; ITX = Iota; CPE = Enterotoxina

A enterotoxina CPE (de *C. perfringens* enterotoxin) é o principal fator de virulência do *C. perfringens* tipo A e corresponde à segunda causa mais comum de intoxicação alimentar e doença gastrointestinal humana no mundo e terceira nos EUA, sendo responsável por 5 a 15% dos casos de diarreia associada a antibioticoterapia, juntamente com o *C. difficile.* A intoxicação alimentar normalmente é autolimitada durando cerca de 24h. Contudo, a bactéria pode causar também infecções invasivas graves que resultam em necrose tecidual que requerem antibioticoterapia e, muitas vezes, debridamento e amputação de um membro (BRYNESTAD; GRANUM, 2002; MCCLANE; CHAKRABARTI, 2004; UZAL et al., 2014).

# 3. OBJETIVOS

## 3.1. Geral:

Investigar a ação antimicrobiana de espécies de *Lactobacillus* e *Bifidobacterium* como potenciais probióticos contra espécies de *Clostridium*.

## 3.2. Específicos:

- Realizar triagem de linhagens de *Bifidobacterium* e *Lactobacillus* capazes de inibir o crescimento de espécies patogênicas de *Clostridium*;
- Caracterizar o potencial probióticos da espécie com maior atividade antimicrobiana através da capacidade de coagregação, adesão à mucina, e tolerância ao pH e sais biliares;
- Determinar o perfil de susceptibilidade aos antibióticos da espécie selecionada.

# 4. RESULTADOS

**4.1 CAPÍTULO 1:** *In vitro* antimicrobial activity and probiotic potential of *Bifidobacterium* and *Lactobacillus* against pathogenic species of *Clostridium*

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# *In vitro* antimicrobial activity and probiotic potential of *Bifidobacterium* and *Lactobacillus* against pathogenic species of *Clostridium*

**Cinara R. A. V. Monteiro [1,2], Monique S. do Carmo [1,2], Bruna O. Melo [2], Matheus S. Alves [2], Camilla I. dos Santos [2], Sílvio G. Monteiro [2,3], Maria Rosa Quaresma Bomfim [2], Elizabeth S Fernandes [2], and Valério Monteiro-Neto [1,2]***

[1] Programa de Pós-graduação em Ciências da Saúde, Universidade Federal do Maranhão, Av. dos Portugueses, campus do Bacanga, São Luís, MA, Brazil; cinaraaragao@hotmail.com (C.R.A.V.M.);
[2] Programa de Pós-graduação, Universidade Ceuma, Rua dos Castanheiros No. 1, jardim Renascença II, São Luís, MA, Brazil; carmo.monique@outlook.com (M.S.C.); brunaoliv.96@gmail.com (B.O.M); matheusjoc@hotmail.com (M.S.A.); camilla_itapary@hotmail.com (C.I.S.); silvio.monteiro@ceuma.br (S.G.M.); mrqbomfim@yahoo.com.br (M.R.Q.B.); elizabeth.soares@ceuma.br (E.S.F.)
[3] Departamento de Biologia, Universidade Federal do Maranhão, Av. dos Portugueses, campus do Bacanga, São Luís, MA, Brazil;

* Correspondence: valerio.monteiro@ceuma.br; Tel.: +55-98-3214-4252

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**Abstract:** *Clostridium* species, mainly *C. butyricum*, *C. difficile*, and *C. perfringens,* are agents of infectious disease resulting from intestinal dysbiosis. In many cases, recurrence can occur after antibiotics, indicating the need for novel therapeutic options that act on the pathogens and also restore the microbiota. Herein, the *in vitro* antimicrobial activity and probiotic potential of clinical and reference strains of *Bifidobacterium* and *Lactobacillus* were investigated against pathogenic species of *Clostridium*. The antimicrobial activity was evaluated by the agar spot test and inhibition of gas production. Then, the probiotic potential of selected strains was assessed by analyzing their coaggregation ability, adhesive properties to host cells and mucin, tolerance to acidic pH and bile salts, and antimicrobial susceptibility profiles. *Lactobacillus plantarum* ATCC 8014 was the most promising strain based on its inhibitory activity against *Clostridium* spp. In addition, this strain met criteria to be considered a probiotic based on its coaggregation ability, adhesive properties, and tolerance to harsh conditions of pH and bile acid salts. Overall, the results indicate that among the studied strains, *L. plantarum* ATCC 8014 presents probiotic potential for controlling infections by the *Clostridium* species studied and should be further evaluated in *in vivo* animal studies.

**Keywords:** probiotics; *Lactobacillus plantarum*; *Clostridium*; dysbiosis; antimicrobial activity

## 1. Introduction

In humans, the normal intestinal microbiota consists of a large number and high diversity of commensal microorganisms (about $10^{13}$ to $10^{14}$), mainly in the large intestine, where they establish a symbiotic relationship that influences the entire host organism [1-3]. Intestinal homeostasis is maintained through complex interactions between the host's immune system and the microbiota [4,5]. However, this mutualistic relationship can be disrupted by a variety of factors, such as changes in diet, use of antibiotics, and immunomodulatory drugs, among others, leading to changes in both bacterial function and diversity, a clinical condition called dysbiosis [6,7].

An imbalance in the intestinal microbiota may cause or contribute to the establishment of infectious and inflammatory diseases such as inflammatory bowel disease [8], antibiotic-associated

diarrhea (AAD) [9], irritable bowel syndrome [10] and necrotizing enterocolitis (NEC) [11]. In addition, there is growing evidence in the literature on the association of dysbiosis with other non-infectious diseases, including type 2 diabetes [12], asthma [13], non-alcoholic fatty liver disease [14], colorectal cancer [15], neurological conditions [16], and cardiovascular diseases [17].

In some infectious disorders of the intestinal tract resulting from dysbiosis, certain specific bacteria have been implicated as etiologic agents, especially potentially pathogenic *Clostridium* species. For example, *Clostridium difficile* is described as one of the leading causes of diarrhea and colitis associated with antibiotic use, with detection frequency ranging from 13% to 28% [9,18]. Mortality rates in patients with *C. difficile* infection may exceed 30%, especially in those individuals experiencing recurrence of infection within 6 months of initial treatment [19].

NEC is the most common and serious intestinal disorder among preterm infants and is diagnosed through radiological findings that include, among other manifestations, the presence of intestinal pneumatosis [20]. Its incidence may reach 12% in children that weigh less than 1 kg at birth [21] and mortality may range from 20 to 50% [22]. Risk factors for NEC include those that affect the normal microbiota, such as neonatal immaturity, enteral feeding, and intestinal colonization [23]. Its etiology is controversial however, and several causative organisms have been proposed, including viruses, *Staphylococcus* spp., various gram-negative bacilli, and *Clostridium* spp. [24]. Recent studies point to *Clostridium butyricum* as an important cause of NEC [25-27], although there are nontoxigenic strains that can be employed as probiotics [28].

*Clostridium perfringens* causes infection in both humans and animals [29]. In humans, like *C. difficile*, under conditions of intestinal microbiota imbalance, *C. perfringens* may similarly be involved in cases of AAD, although with a lower prevalence [9,30,31]. Depending on the toxigenic type, *C. perfringens* may also cause other disorders in the intestinal tract, such as food poisoning and necrotic enteritis, in addition to tissue infections accompanied by myonecrosis, such as gas gangrene due to trauma [29].

Antibiotic therapy is the first line of treatment for these infectious intestinal disorders. However, recurrence is frequent, particularly in cases of AAD, since the microbiota remains unbalanced due to the use of broad-spectrum antibiotics [9,19]. Alternative interventions have been employed, such as narrow-spectrum antibiotics, dietary changes, fecal transplantation, and probiotics, which may attenuate the clinical symptoms of dysbiosis, restore diversity of the intestinal microbiota, and improve host health [32-34].

Some studies have shown alleviation of AAD [35,36] and NEC [37,38] with probiotics. The efficacy of distinct probiotic strains, especially among species of *Bifidobacterium* and *Lactobacillus*, suggests that they may have common properties that could positively impact patient health in these infections [32,39]. However, the basis of these properties is not yet fully understood [39] and there are no compelling explanations for the effects of probiotics in AAD or NEC. Several plausible mechanisms have been investigated and may contribute to the observed health benefits, but in terms of translational research, this is an evident shortcoming that hinders the development of improved therapies.

Therefore, the objective of this study was to carry out an *in vitro* screening of clinical and reference strains of *Bifidobacterium* and *Lactobacillus* with antimicrobial activity against *C. butyricum*, *C. difficile*, and *C. perfringens*. The most promising strain was subjected to analysis of criteria for consideration as a potential probiotic, including its ability to adhere to eukaryotic cells and mucus and its tolerance to acidic pH and bile salts.

## 2. Materials and Methods

### *2.1 Bacterial strains and growth conditions*

The following *Bifidobacterium* and *Lactobacillus* reference strains were studied: *Bifidobacterium longum* subsp. *longum* ATCC 15707, *Lactobacillus brevis* ATCC 367, *Lactobacillus delbrueckii* subsp. *delbrueckii* ATCC 9649, *Lactobacillus fermentum* ATCC 23271, *Lactobacillus paracasei* subsp. *paracasei* ATCC 335, *Lactobacillus plantarum* ATCC 8014, and *Lactobacillus rhamnosus* ATCC 9595, which were

obtained from the National Institute of Quality Control in Health (INCQS, FIOCRUZ, Rio de Janeiro, Brazil). *Lactobacillus rhamnosus* GG (LGG, ATCC 53103) was isolated from a commercial probiotic product (Floridral - Pharmaforce ApS, Copenhagen, Denmark) and used as a positive control. In addition, fecal isolates from newborn infants were evaluated and maintained in the Culture Collection Sector of Ceuma University, including *Bifidobacterium longum* 49.3, *Bifidobacterium animalis* subsp. *lactis* 56.1, *Bifidobacterium bifidum* 14.2, and *Lactobacillus fermentum* 54.2. All *Bifidobacterium* and *Lactobacillus* isolates were routinely cultured on agar or MRS broth (Man-Rogosa-Sharpe, Difco-BD, Detroit, MI, USA) with 0.25% L-cysteine and incubated at 37 °C for 24–48 h under anaerobic conditions. Cultures were stored in MRS broth with 20% glycerol at –80 °C.

*C. butyricum* ATCC 860, *C. difficile* ATCC 9689, and *C. perfringens* ATCC 12924 were obtained from INCQS (FIOCRUZ, Rio de Janeiro, Brazil). *Clostridium* strains were cultured in RCM (reinforced clostridial medium, Acumedia, Lansing, MI, USA) or thioglycolate medium (Acumedia) and incubated at 37 °C for 24–48 h in an anaerobic atmosphere. They were stored in RCM with 20% glycerol at -80 °C.

### *2.2 Antimicrobial activity screening*

The ability of potential probiotics to inhibit *Clostridium* growth was evaluated in two distinct assays. All assays were performed in triplicate over three days.

#### 2.2.1. Agar spot test

The agar spot test procedure was performed as described previously [40], with modifications. Briefly, in a Petri dish containing 10 mL of MRS agar, 5 µL of each probiotic culture was spotted onto one quadrant of the culture medium, followed by incubation at 37 °C for 24 h under anaerobic conditions. After incubation, 10 mL of thioglycolate agar was overlaid onto the MRS agar containing the growth of *Bifidobacterium* and *Lactobacillus* strains. After solidification of the culture medium at room temperature (25 – 28 °C), *Clostridium* spp. suspensions (McFarland standard No. 0.5, $1.5 \times 10^8$ colony-forming units per milliliter - CFU/mL) were spread with the aid of a swab. The plates were incubated at 37 °C for 24 h under anaerobic conditions. The formation of a clear halo around growth of the probiotics was indicative of antimicrobial activity. The diameter of the growth inhibition halo was measured and expressed in millimeters.

#### 2.2.2 Inhibition of gas production

The ability of probiotics to inhibit the growth of *Clostridium* strains was also evaluated by assessing the inhibition of gas production due to the fermentative action of the pathogens, as described previously [41,42], with some modifications. Briefly, the assay was performed by inoculating 1 µL (~$10^7$ UFC) of *Clostridium* culture into the upper third of the RCM agar layer (supplemented with 1.5 g/100 mL bacteriological agar), composed of 3 mL per tube. Subsequently, 3 mL of MRS containing 0.7 g% bacteriological agar was melted, cooled to 50 °C, and inoculated with 30 µL (~$10^8$ UFC) of each probiotic culture. The contents were homogenized by vortexing and immediately poured over the RCM agar layer in tubes inoculated with the *Clostridium* strains. RCM agar with *Clostridium* and MRS agar without inoculated probiotics were used as negative controls. The tubes were incubated under anaerobic conditions at 37 °C for 24 h. The assays were performed in triplicate, with and without buffering of the MRS medium with $K_2HPO_4$ and $KH_2PO_4$ (100 mM) to verify whether or not the inhibition was due to acid production by the probiotics. A positive assay for antimicrobial activity was characterized by the absence of gas production, that is, when bubbles were not formed inside the culture media or medium breakage did not occur.

140 *2.3 Coaggregation test*

141 After cultivation of the probiotic strains and *Clostridium* species, aliquots of 1 mL of each culture
142 were washed twice with phosphate-buffered saline (PBS, pH 7.2), centrifuged at 5,000 × *g* for 15 min,
143 and resuspended in PBS. The optical density of each suspension was adjusted ($OD_{620}$ nm = 0.1), and
144 500-µL aliquots of the probiotic suspensions were mixed with 500 µL of each pathogen suspension
145 in 24-well plates (Nunc, Roskilde, Denmark) and incubated at 37 °C for 4 h under constant stirring
146 (100 rpm) on an orbital shaker. Plates were observed for macroscopically visible clumps and under
147 inverted microscopy [43]. Glass slides were also prepared with 5 µL of each suspension and evaluated
148 under the microscope for visualization of bacterial coaggregates after Gram staining. *L. fermentum*
149 ATCC 23271 was used as a positive control in this assay, since high coaggregation scores were
150 previously demonstrated [44]. Control assays were performed with individual bacterial samples to
151 assess their ability to autoaggregate.

152 *2.4 Mucin binding assay*

153 The ability of selected probiotic strains to bind to mucin was evaluated essentially as described
154 by Tallon et al. [45]. A volume of 100 µL of a 10 mg/mL mucin solution in PBS (pH 7.2) was added to
155 the wells of polystyrene microtiter plates (Nunc) and incubated overnight at 4 °C. The wells were
156 washed twice with 200 µL PBS and saturated with a 2% (w/v) bovine serum albumin (BSA) solution
157 (Sigma-Aldrich, St. Louis, MO, USA) for 4 h at 4 °C. Finally, the wells were washed twice with 200
158 µL PBS. At least four replicates were used to estimate the adhesion of a given strain. Probiotic cultures
159 in MRS broth were washed three times in PBS, and the final suspension was standardized by
160 spectrophotometry ($OD_{600nm}$ = 0.1). Aliquots of 100 µL of the bacterial suspension were added to each
161 well, and the microplates were incubated at 37 °C for 1 h. After this, the wells were washed 12 times
162 with 1 mL PBS to remove non-adherent bacteria. The wells were treated with 200 µL of 0.5% Triton
163 X-100 (Sigma-Aldrich), and the plates were then incubated for 2 h at room temperature under orbital
164 shaking to release the adhered bacteria. Then, the wells were scraped with a sterile tip, and the
165 number of bacteria with binding ability to mucin was estimated by serial decimal dilutions in PBS
166 and plating on MRS agar, followed by incubation at 37 °C for 24 h under anaerobic conditions. *L.*
167 *fermentum* ATCC 23271 was used as a positive control [44].

168 *2.5 Adhesion to eukaryotic cells*

169 Adhesion to HeLa was evaluated according to the method of Carmo et al [44]. A 300-µL aliquot
170 of each potential probiotic cultured in MRS broth was washed three times with PBS (pH 7.4, Sigma-
171 Aldrich), and the bacterial pellet was resuspended in 300 µL Dulbecco's modified Eagle's medium
172 (DMEM, Sigma-Aldrich). Monolayers of HeLa cells grown in 24-well microplates (Nunc) containing
173 DMEM supplemented with 10% fetal bovine serum (Gibco, Gaithersburg, MD, USA), with or without
174 glass coverslips, were inoculated with 50 µL (~$2.3 \times 10^7$ CFU) of bacterial suspension and incubated
175 at 37 °C under 5% $CO_2$ for 3 h. Then, each well was washed three times with PBS to remove non-
176 adherent bacteria. For quantification of the adherent bacteria, the HeLa cell monolayers in the wells
177 without coverslips were treated with 1 mL of 0.1% Triton X-100 (Sigma-Aldrich) for 5 min and
178 scraped with the aid of a tip. Thereafter, serial decimal dilutions were spread on MRS agar plates and
179 incubated at 37 °C for 24 h. The total number of bacteria adhered to the cells was expressed as CFU
180 per milliliter. Visualization of bacterial adherence to eukaryotic cells was performed after fixation
181 with methanol (Amresco, Gymea, Australia) and staining with May-Grunwald and Giemsa
182 (Amresco). Gram staining was also used to better visualize gram-positive *Lactobacillus* adhered to the
183 cells. The coverslips were then mounted on glass slides and visualized by light microscopy under a
184 100× oil immersion objective.
185

186 *2.6 Tolerance to acidic pH and bile salts*

187 Tolerance of selected bacteria to acidic pH (2 and 4) and bile salts (0.5% and 1%, Oxgall, Sigma-
188 Aldrich) was evaluated as previously described, with minor modifications [46]. Briefly, 900 μL MRS,
189 adjusted to pH 2 or 4 or non-adjusted (control) or supplemented with 0, 0.5, or 1.0% (w/v) Oxgall
190 (Sigma-Aldrich), was inoculated with 100 μL of a 24-h culture, which had been previously washed
191 three times with PBS and resuspended in the same volume of MRS broth. After incubation at 37 °C
192 for 3 h under anaerobic conditions, the percentage of viable bacteria relative to that in the control was
193 determined by plate counting on MRS agar.

194 *2.7 Antibiotic susceptibility testing*

195 Antibiotic susceptibility of probiotics was determined by a modification of the agar overlay
196 diffusion method, as previously described [47]. Commercial discs (Oxoid) containing different
197 antibiotics, including ciprofloxacin (5 μg), clindamycin (2 μg), chloramphenicol (30 μg),
198 erythromycin (15 μg), gentamicin (10 μg), penicillin (10 μg), rifampicin (5 μg), co-trimoxazole (25 μg),
199 vancomycin (30 μg), and tetracycline (30 μg), were placed on MRS agar plates inoculated with
200 *Lactobacillus* ($10^8$ CFU/mL). The plates were incubated anaerobically at 37 °C for 24 h. Antibiotic
201 susceptibility was evaluated based on the diameter (in millimeters) of the growth inhibition zone
202 around the discs [47]. The reference strain *Staphylococcus aureus* ATCC 25923 was used for quality
203 control of antibiotic discs and tested in Mueller-Hinton agar, as recommended [48].

204 *2.8 Statistical analyses*

205 Statistical analyses were performed using NCSS 11 Statistical Software (2016; NCSS, Kaysville,
206 UT, USA). Adherence to eukaryotic cells and to mucin was expressed as log CFU/mL (± SD).
207 Tolerance to acidic pH and to bile salts was compared to growth in standard MRS medium. The
208 Shapiro-Wilk test was carried out and confirmed that all variables were normally distributed. Thus,
209 all comparisons were carried out by the Student *t*-test. Statistical significance was established at $p <$
210 0.05. All assays were performed in three independent experiments conducted on three different days.

211 **3. Results**

212 *3.1. Selection of strains with antimicrobial activity against* Clostridium *spp.*

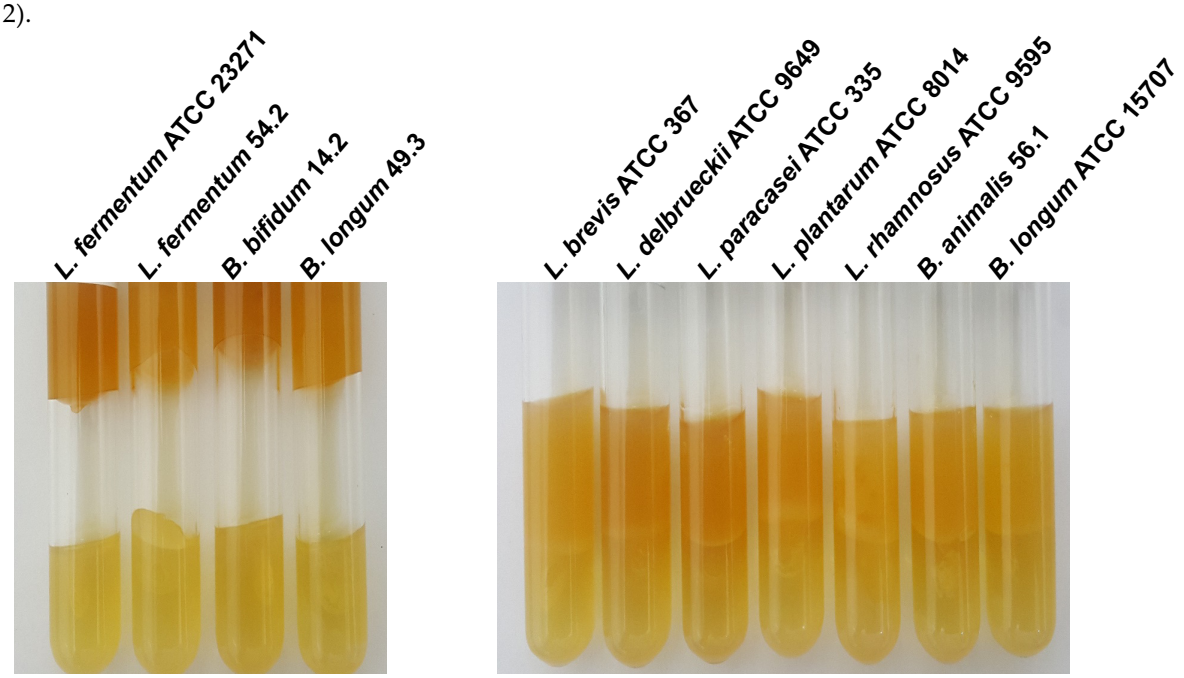
213 The antimicrobial effects of potential probiotics, as determined by the agar spot test, are shown
214 in Table 1. Eleven strains (91.7%) were able to inhibit *C. butyricum*, whereas *C. difficile* and *C.*
215 *perfringens* were inhibited by 9 (75%) strains each. The diameter of the inhibition zone varied among
216 the clinical and reference probiotic strains. Of the 12 species tested, 8 (66.7%) exhibited antimicrobial
217 activity against all three *Clostridium* species. The largest inhibition zones were produced by *L.*
218 *plantarum* ATCC 8014, mainly against *C. butyricum* (17 mm). Only *B. animalis* 56.1 showed no
219 inhibitory activity against any *Clostridium* species in this assay.

220

**Table 1.** Inhibitory activity of *Bifidobacterium* and *Lactobacillus* against *Clostridium* spp. based on the agar spot test.

| Potential probiotics | Diameter of inhibition zones (mm ± SD) of: | | |
|---|---|---|---|
| | *C. butyricum* | *C. difficile* | *C. perfringens* |
| *B. animalis* 56.1 | 0 | 0 | 0 |
| *B. bifidum* 14.2 | 12 (1.6) | 12 (0.4) | 11 (1.7) |
| *B. longum* ATCC 15707 | 11 (0.4) | 12 (0.2) | 11 (1.4) |
| *B. longum* 49.3 | 12 (0.4) | 11 (0.0) | 10 (0.7) |
| *L. brevis* ATCC 367 | 9 (0.4) | 0 | 0 |
| *L. delbrueckii* ATCC 9649 | 10 (1.5) | 11 (0.7) | 12 (2.4) |
| *L. fermentum* ATCC 23271 | 10 (1.1) | 10 (0.5) | 10 (0.6) |
| *L. fermentum* 54.2 | 13 (0.7) | 9 (0,4) | 10 (0.0) |
| *L. paracasei* ATCC 335 | 11 (0.5) | 12 (0.3) | 11 (0.4) |
| *L. plantarum* ATCC 8014 | 17 (0.8) | 13 (1.1) | 13 (0.6) |
| *L. rhamnosus* ATCC 9595 | 11 (0.0) | 0 | 0 |
| *L. rhamnosus* GG ATCC 53103 | 10 (0.9) | 0 | 0 |

To assess whether or not the inhibitory activity on *Clostridium* spp. was due to acid produced by the probiotics, a test was conducted to evaluate the inhibition of gas production by *C. butyricum* ATCC 860 in culture medium. This test was carried out in MRS medium with and without phosphate buffer. *C. butyricum* ATCC 860 was selected because this strain usually produces a large amount of gas resulting from its fermentative activity in thioglycolate medium or RCM. In the presence of buffer, the strains *L. brevis* ATCC 367, *L. delbrueckii* ATCC 9649, *L. paracasei* ATCC 335, *L. plantarum* ATCC 8014, *L. rhamnosus* ATCC 9595, *B. animalis* 56.1, and *B. longum* ATCC 15707 inhibited gas production by *Clostridium* spp., whereas the other strains did not (Figure 1). Among the strains that inhibited gas production in the absence of the phosphate buffer, only *B. bifidum* 14.2 gave a negative result when the assay was carried out in the of presence phosphate buffer in the MRS medium (Table 2).




**(a)** **(b)**

246

247 **Figure 1.** Inhibition of gas production. The lower layer corresponds to the RCM agar inoculated with
248 *Clostridium butyricum* ATCC 860, and the upper layer is MRS medium with 0.7% agar and 100 mM phosphate
249 buffer inoculated with *Bifidobacterium* and *Lactobacillus* strains; cultures were incubated at 37 °C for 24 h under
250 anaerobic conditions. (a) Gas production by *C. butyricum* in buffered MRS medium, indicating the absence of
251 inhibitory activity of *Lactobacillus* and *Bifidobacterium* strains; (b) Inhibitory activity of five *Lactobacillus* and two
252 *Bifidobacterium* strains on gas production by *Clostridium butyricum*.

253

254 **Table 2.** Inhibition of gas production by *C. butyricum* induced by probiotics grown in MRS medium
255 with and without phosphate buffer.

| Strains | Without buffer | With buffer |
|---|---|---|
| *B. animalis* 56.1 | + | + |
| *B. bifidum* 14.2 | + | − |
| *B. longum* ATCC 15707 | + | + |
| *B. longum* 49.3 | − | − |
| *L. brevis* ATCC 367 | + | + |
| *L. delbrueckii* ATCC 9649 | + | + |
| *L. fermentum* ATCC 23271 | − | − |
| *L. fermentum* 54.2 | − | − |
| *L. paracasei* ATCC 335 | + | + |
| *L. plantarum* ATCC 8014 | + | + |
| *L. rhamnosus* ATCC 9595 | + | + |

256

257

258 *3.2.* L. plantarum *ATCC 8014 presents probiotic potential*

259 *L. plantarum* ATCC 8014, the strain with the highest antimicrobial activity in the agar spot test
260 for the three *Clostridium* strains, was selected for further analysis and characterization as a potential
261 probiotic. In terms of its coaggregation capacity, we observed that *L. plantarum* ATCC 8014 interacted
262 clearly with the pathogens, forming bacterial aggregates with the three species of *Clostridium* (Figure
263 2).

264 Investigation of the adhesion properties of *L. plantarum* ATCC 8014 revealed its ability to interact
265 with eukaryotic cells, as demonstrated in the assay with HeLa cells (Figure 3). In fact, adherence
266 values for *L. plantarum* ATCC 8014 were higher than those observed for *L. fermentum* ATCC 23271
267 (Table 3, $P = 0.0037$). In contrast, *L. fermentum* ATCC 23271 exhibited a greater ability to bind mucin
268 than *L. plantarum* ATCC 8014 (Table 3, $P < 0.0001$).

269

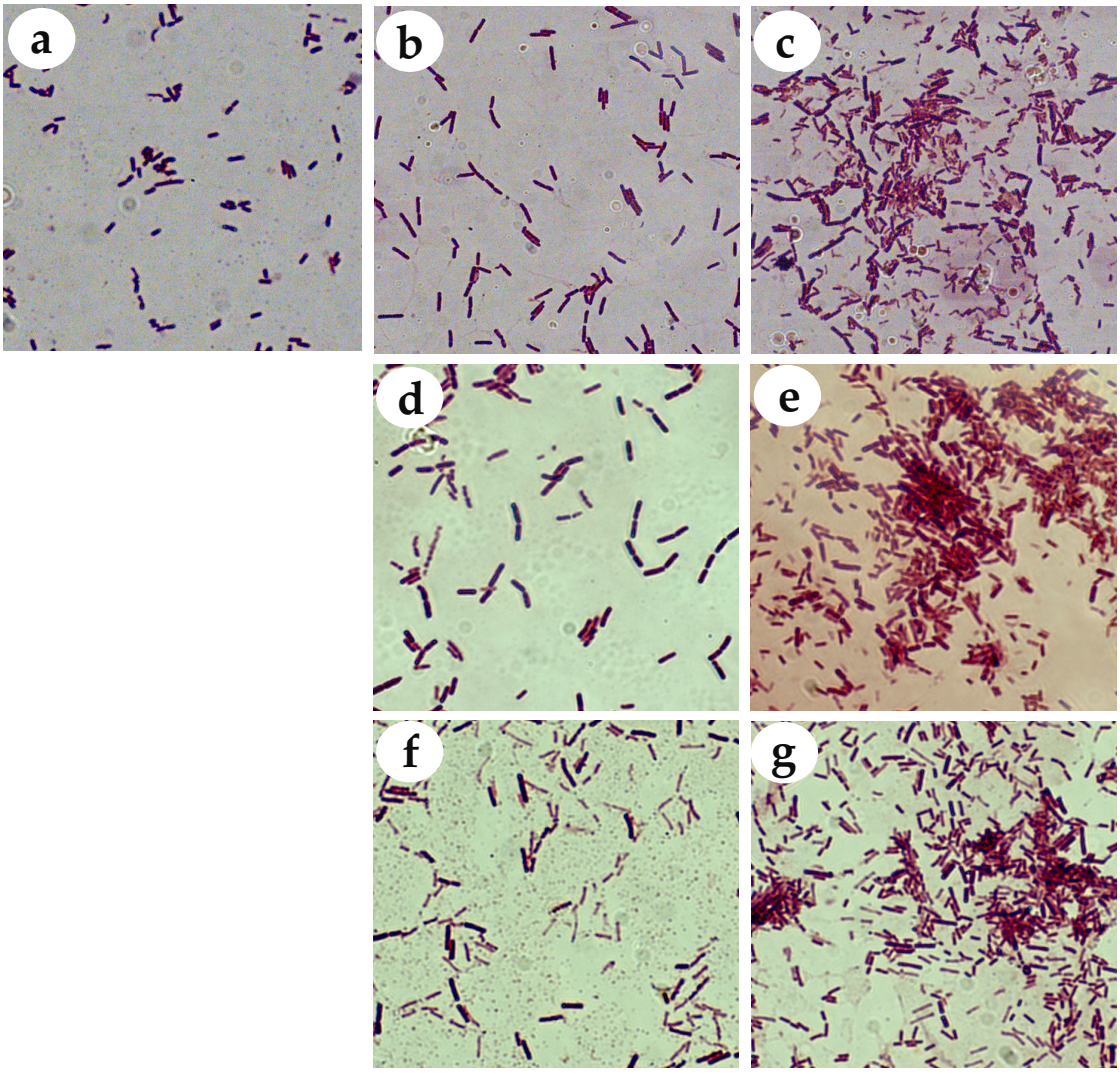



**Figure 2.** Coaggregation of *L. plantarum* ATCC 8014 with the three species of *Clostridium*. (a) *L. plantarum*; (b) *C. butyricum*; (c) Coaggregation of *L. plantarum* and *C. butyricum*; (d) *C. difficile*; (e) Coaggregation of *L. plantarum* and *C. difficile*; (f) *C. perfringens*; (g) Coaggregation of *L. plantarum* and *C. perfringens*.

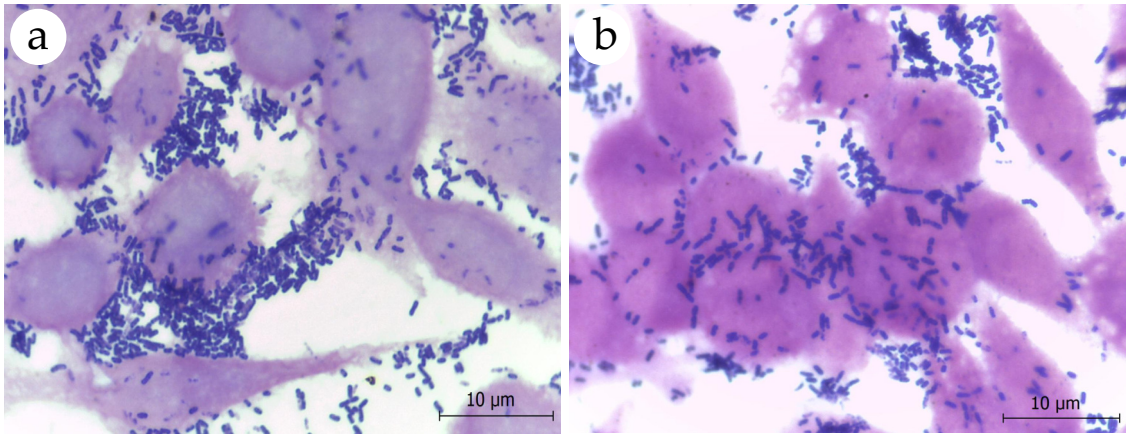



**Figure 3.** Adhesion assay of (a) *L. plantarum* ATCC 8014 and (b) *L. fermentum* ATCC 23271 to HeLa cells after inoculation of approximately $10^7$ CFU of bacterial suspensions. Cell monolayers were Gram-stained.

299

300 **Table 3.** Adherence properties of *L. plantarum* ATCC 8014 to eukaryotic cells and to mucin in comparison with
301 the control

| Assays [1] | *L. plantarum* ATCC 8014 | *L. fermentum* ATCC 23271 | *p* Value [2] |
|---|---|---|---|
| Cell adhesion | 7.602 (±0.135) | 7.349 (±0.053) | 0.0037 |
| Mucin binding | 5.057 (±0.062) | 5.370 (±0.031) | < 0.0001 |

302 [1] Data are expressed as mean $\log_{10}$ CFU/mL (standard deviations) of triplicate experiments performed on three
303 independent days. [2] Comparative analysis was performed by Student's *t*-test ($p < 0.05$).

304

305 *L. plantarum* ATCC 8014 exhibited growth at pH 2 and 4 after 180 min of exposure, with growth
306 values of 70.3% and 97.8%, respectively, relative to that observed in standard MRS medium (Table
307 4). Assays performed at bile salt concentrations of 0.5% and 1.0% resulted in growth values of 110.8%
308 and 90.1%, respectively, relative to that observed in standard medium. No differences were observed
309 relative to the growth of *L. fermentum* strain ATCC 23271 (Table 4).

310

311 **Table 4.** Survival of *L. plantarum* ATCC 8014 and *L. fermentum* ATCC 23271 in the presence of acidic pH and
312 bile salts

| Conditions | % Survival (± SD)[1] | | *p* value[2] |
|---|---|---|---|
| | *L. plantarum* | *L. fermentum* | |
| pH 2.0 | 70.3 (± 4.46) | 64.7 (± 6.49) | 0.1155 |
| pH 4.0 | 97.8 (± 5.67) | 106.2 (± 9.36) | 0.7606 |
| Bile salts 0.5% | 110.8 (12.04) | 112.7 (± 8.79) | 0.0912 |
| Bile salts 1.0% | 90.1 (± 3.77) | 92.6 (± 3.07) | 0.2419 |

313 [1] Data represent survival percentage of microorganisms after 180 min of exposure to distinct conditions in
314 comparison to bacterial growth of each under standard conditions. [2] Comparative analysis was performed by
315 the Student's *t*-test ($p < 0.05$).

316

317

318 The antibiotic susceptibility profile of *L. plantarum* ATCC 8014 was evaluated using the
319 overlay diffusion method. *L. plantarum* ATCC 8014 showed resistance to ciprofloxacin and
320 vancomycin, and sensitivity to all other antibiotics tested (Table 5).

321

322 **Table 5.** Antimicrobial susceptibility of *L. plantarum* ATCC 8014 according to agar overlay diffusion

| Results | Antibiotics |
|---|---|
| Susceptible | Clindamycin |
| | Chloramphenicol |
| | Erythromycin |
| | Gentamicin |
| | Penicillin |
| | Rifampicin |
| | Tetracycline |
| Moderately susceptible | Co-trimoxazole |
| Resistant | Ciprofloxacin |
| | Vancomycin |

323

324 **4. Discussion**

325 Some probiotic strains have been successfully used in clinical studies for the treatment or
326 prevention of DAA [35,36] and EN [37,38]. However, their mechanisms of action remain unclear. It
327 can be argued that this is not important, since the positive effects of probiotic interventions are
328 generally observed with some reliability. However, controversial findings on AAD and NEC
329 interventions have been reported in the literature, as a positive effect does not always occur when
330 used in cases of dysbiosis [49]. A better understanding of the relevant "central probiotic properties"
331 that contribute to their inhibitory effects on major etiological agents of these clinical syndromes could
332 therefore aid in the design of a more rational strategy for selecting and producing more effective
333 probiotics [32].

334 In this study, we showed that different clinical and reference species present different levels of
335 antimicrobial efficacy against *C. butyricum*, *C. difficile,* and *C. perfringens*. Evaluation of the
336 antimicrobial activity of 12 potential probiotic bacteria, including four species of *Bifidobacterium* and
337 eight species of *Lactobacillus*, indicated that *L. plantarum* strain ATCC 8014 exhibited the greatest
338 capacity of inhibiting the growth of the three reference strains of *Clostridium*, based on the activity
339 detected using screening methods. In addition to *L. plantarum* ATCC 8014, other species showed
340 inhibitory activity against one or all *Clostridium* strains. However, their antimicrobial activities were
341 evidenced with lower zones of inhibition or were variable in comparison to the inhibition test of gas
342 production. This variability in performance has previously been reported and suggests that more
343 than one method should be used to assess the antimicrobial activity of probiotics, given that the
344 conditions of each methodology may interfere with the results [50].

345 Various species of *Lactobacillus* are able to produce compounds with antimicrobial activities,
346 including organic (acetic and lactic) acids, low-molecular-weight compounds, antifungal peptides,
347 and antibacterial peptides (bacteriocins) [51,52]. It appears however that the inhibition of *Clostridium*
348 growth exhibited by the probiotic strains was not the result of the overproduction of acids, since the
349 addition of buffer to the MRS media did not affect the inhibitory activity of the majority of these
350 species. Furthermore, some species of *Clostridium* exhibit intense fermentative activity, resulting in
351 the production of large quantities of organic acids, including acetic, lactic, formic, butyric, and
352 propionic acid, among other substances; thus, they would likely already be habituated to these and
353 would survive acidic pH conditions [52,53].

354 Therefore, it is most likely that other molecules produced by the probiotics are involved in the
355 inhibition of *Clostridium* strains. Recently, it has been demonstrated that *Lactobacillus* metabolites

isolated from vaginal smears exerted *in vitro* bacteriostatic effects against *C. perfringens* in adult vaginal swabs [54]. This activity has been associated to the production of protein compounds such as bacteriocins, which are produced by several species of probiotics and have bactericidal or bacteriostatic actions. Bacteriocins may increase the permeability of the inner membrane of bacteria, thus contributing to their rupture and interfering with bacterial cell wall synthesis, resulting in pore formation by binding to the peptidoglycan precursor lipid [55]. Lacticin 3147, for example, produced by *Lactococcus lactis*, forms selective pores in the cell walls of some pathogenic gram-positive bacteria, including *C. difficile*, resulting in its death [56].

The nature of the compound(s) produced by *L. plantarum* ATCC 8014 and its potential bactericidal or bacteriostatic actions are not yet known. However, even if such compounds have bacteriostatic action or are capable of inducing only sporulation, such action could be relevant to controlling the clinical manifestation of infections caused by *Clostridium*, neutralizing the metabolic activity of the pathogen and, consequently, the production of toxins and other virulence factors involved. In addition, bacteriocins have a more targeted inhibitory action against gram-positive bacteria [50,52].

Several criteria are used to define microorganisms as probiotics, including the ability to: 1) coaggregate with microbial pathogens; 2) adhere to eukaryotic cells and mucus; and 3) tolerate conditions of acidic pH and bile salts, among other properties [57,58]. *L. plantarum* ATCC 8014 fulfilled these criteria to be considered a potential probiotic, as it tolerated acidic pH and bile salts under the conditions tested, coaggregated with *Clostridium* spp., and exhibited adhesive properties suggesting its capacity for *in vivo* colonization. Although it demonstrated lower mucin binding than the *L. fermentum* strain ATCC 23271, the results of the binding assay were still high.

A worrying issue in the selection of probiotics is the potential transmissibility of drug resistance exhibited by probiotic bacteria to be used in foods or supplements, especially if the microorganism in question carries plasmids [59,60]. In this study, *L. plantarum* ATCC 8014 showed resistance to ciprofloxacin and vancomycin. However, despite having plasmids [61], resistance to these antibiotics is considered intrinsic and non-transmissible in this species [62,63].

Ingestion of non-pathogenic bacterial species, such as probiotics, may contribute to important protective functions by reducing paracellular permeability, providing an innate defense against pathogens and a physical impediment in the form of the mucosal layer, which may help protect against infection, prevent inflammation, and maintain mucosal integrity. In addition, there is a large panel of probiotic strains that are currently in use. However, in most cases, probiotic action has been shown to be species-specific or even strain-specific [32,64 70,65,66]. Thus, the possibility of a potential probiotic with proven antimicrobial action against multiple pathogenic species of *Clostridium*, which are commonly associated with pathologies resulting from an imbalance in the microbiota, represents a significant advance for the reduction of morbidity and mortality rates arising from these clinical syndromes. In addition to being a more rational therapeutic approach, use of a probiotic would not have a negative impact on the intestinal microbiota and would not exert pressure for the selection of resistant bacteria, as with conventional antibiotic therapy [67].

## 5. Conclusion

Our findings allow us to conclude that *L. plantarum* strain ATCC 8014 has probiotic potential, with antimicrobial activity against *C. butyricum* ATCC 860, *C. difficile* ATCC 9689, and *C. perfringens* ATCC 12924. Additionally, this microorganism fulfills essential criteria for status as 'generally recognized as safe' (GRAS). However, *in vivo* studies are needed for a better assessment of its efficacy and safety.

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# *ANEXOS*

**ANEXO 1** – Normas da revista *NUTRIENTS* para publicação de artigo





## Journal Menu


# *Nutrients* — Instructions for Authors

Shortcuts

- Manuscript Submission Overview

## Submission Checklist

Please.

1. read the Aims & Scope (/journal/nutrients/about) to gain an overview and assess if your manuscript is suitable for this journal;
2. use the Microsoft Word template (/files/word-templates/nutrients-template.dot) or LaTeX template (https://www.mdpi.com/authors/latex) to prepare your manuscript;
3. make sure that issues about publication ethics, research ethics, copyright (https://www.mdpi.com/authors/rights), authorship, figure formats, data and references format have been appropriately considered;
4. ensure that all authors have approved the content of the submitted manuscript.

## Manuscript Submission Overview

### Types of Publications

*Nutrients* has no restrictions on the length of manuscripts, provided that the text is concise and comprehensive. Full experimental details must be provided so that the results can be reproduced. *Nutrients* requires that authors publish all experimental controls and make full datasets available where possible (see the guidelines on Supplementary Materials and references to unpublished data).

Manuscripts submitted to *Nutrients* should neither been published before nor be under consideration for publication in another journal. The main article types are as follows:

- *Articles:* Original research manuscripts. The journal considers all original research manuscripts provided that the work reports scientifically sound experiments and provides a substantial amount of new information. Authors should not unnecessarily divide their work into several related manuscripts, although Short *Communications* of preliminary, but significant, results will be considered. Quality and impact of the study will be considered during peer review.
- *Reviews:* These provide concise and precise updates on the latest progress made in a given area of research. Systematic reviews should follow the PRISMA guidelines. (https://www.mdpi.com/editorial_process#standards)
- *Case reports:* Case reports present detailed information on the symptoms, signs, diagnosis, treatment (including all types of interventions), and outcomes of an individual patient. Case reports usually describe new or uncommon conditions that serve to enhance medical care or highlight diagnostic approaches.

### Submission Process

Manuscripts for *Nutrients* should be submitted online at susy.mdpi.com (https://susy.mdpi.com) The submitting author, who is generally the corresponding author, is responsible for the manuscript during the submission and peer-review process. The submitting author must ensure that all eligible co-authors have been included in the author list (read the criteria to qualify for authorship) and that they have all read and approved the submitted version of the manuscript. To submit your manuscript, register and log in to the submission website (https://susy.mdpi.com). Once you have registered, click here to go to the submission form for *Nutrients* (https://www.mdpi.com/user/manuscripts/upload/?journal=nutrients). All co-authors can see the manuscript details in the submission system, if they register and log in using the e-mail address provided during manuscript submission.

### Accepted File Formats

Authors must use the Microsoft Word template (../../../files/word-templates/nutrients-template.dot) or LaTeX template (https://www.mdpi.com/authors/latex) to prepare their manuscript. Using the template file will substantially shorten the time to complete copy-editing and publication of accepted manuscripts. The total amount of data for all files must not exceed 120 MB. If this is a problem, please contact the editorial office nutrients@mdpi.com. Accepted file formats are:

- *Microsoft Word:* Manuscripts prepared in Microsoft Word must be converted into a single file before submission. When preparing manuscripts in Microsoft Word, the *Nutrients* Microsoft Word template file (https://www.mdpi.com/files/word-templates/nutrients-template.dot) must be used. Please insert your graphics (schemes, figures, *etc.*) in the main text after the paragraph of its first citation.
- *LaTeX:* Manuscripts prepared in LaTeX must be collated into one ZIP folder (include all source files and images, so that the Editorial Office can recompile the submitted PDF). When preparing manuscripts in LaTeX, please use the *Nutrients* LaTeX template files (https://www.mdpi.com/authors/latex). You can now also use the online application writeLaTeX (http://www.writelatex.com) to submit articles directly to *Nutrients*. The MDPI LaTeX template file should be selected from the writeLaTeX template gallery (https://www.writelatex.com/templates/mdpi-article-template/fvjngfxymnbr).
- *Supplementary files:* May be any format, but it is recommended that you use common, non-proprietary formats where possible (see below for further details).

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A cover letter must be included with each manuscript submission. It should be concise and explain why the content of the paper is significant, placing the findings in the context of existing work and why it fits the scope of the journal. Confirm that neither the manuscript nor any parts of its content are currently under consideration or published in another journal. Any prior submissions of the manuscript to MDPI journals must be acknowledged. The names of proposed and excluded reviewers should be provided in the submission system, not in the cover letter.

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This journal automatically deposits papers to PubMed Central after publication of an issue. Authors do not need to separately submit their papers through the NIH Manuscript Submission System (NIHMS, http://nihms.nih.gov/ (http://www.nihms.nih.gov/)).

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# Manuscript Preparation

## General Considerations

- **Research manuscripts** should comprise:
  - Front matter: Title, Author list, Affiliations, Abstract, Keywords
  - Research manuscript sections: Introduction, Materials and Methods, Results, Discussion, Conclusions (optional).
  - Back matter: Supplementary Materials, Acknowledgments, Author Contributions, Conflicts of Interest, References.
- **Review manuscripts** should comprise the front matter, literature review sections and the back matter. The template file can also be used to prepare the front and back matter of your review manuscript. It is not necessary to follow the remaining structure. Structured reviews and meta-analyses should use the same structure as research articles and ensure they conform to the PRISMA (https://www.mdpi.com/editorial_process) guidelines.
- **Case reports** should include a succinct introduction about the general medical condition or relevant symptoms that will be discussed in the case report; the case presentation including all of the relevant de-identified demographic and descriptive information about the patient(s), and a description of the symptoms, diagnosis, treatment, and outcome; a discussion providing context and any necessary explanation of specific treatment decisions; a conclusion briefly outlining the take-home message and the lessons learned.
- **Graphical abstract:** Authors are encouraged to provide a graphical abstract as a self-explanatory image to appear alongside with the text abstract in the Table of Contents. Figures should be a high quality image in any common image format. Note that images displayed online will be up to 11 by 9 cm on screen and the figure should be clear at this size.
- **Abbreviations** should be defined in parentheses the first time they appear in the abstract, main text, and in figure or table captions and used consistently thereafter.
- **SI Units** (International System of Units) should be used. Imperial, US customary and other units should be converted to SI units whenever possible
- **Accession numbers** of RNA, DNA and protein sequences used in the manuscript should be provided in the Materials and Methods section. Also see the section on Deposition of Sequences and of Expression Data.
- **Equations:** If you are using Word, please use either the Microsoft Equation Editor or the MathType add-on. Equations should be editable by the editorial office and not appear in a picture format.
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- **Preregistration:** Where authors have preregistered studies or analysis plans, links to the preregistration must be provided in the manuscript.
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## Front Matter

These sections should appear in all manuscript types

- **Title:** The title of your manuscript should be concise, specific and relevant. It should identify if the study reports (human or animal) trial data, or is a systematic review, meta-analysis or replication study. When gene or protein names are included, the abbreviated name rather than full name should be used.
- **Author List and Affiliations:** Authors' full first and last names must be provided. The initials of any middle names can be added. The PubMed/MEDLINE standard format is used for affiliations: complete address information including city, zip code, state/province, country, and all email addresses. At least one author should be designated as corresponding author, and his or her email address and other details should be included at the end of the affiliation section. Please read the criteria to qualify for authorship.
- **Abstract:** The abstract should be a total of about 200 words maximum. The abstract should be a single paragraph and should follow the style of structured abstracts, but without headings: 1) Background: Place the question addressed in a broad context and highlight the purpose of the study; 2) Methods: Describe briefly the main methods or treatments applied. Include any relevant preregistration numbers, and species and strains of any animals used. 3) Results: Summarize the article's main findings; and 4) Conclusion: Indicate the main conclusions or interpretations. The abstract should be an objective representation of the article: it must not contain results which are not presented and substantiated in the main text and should not exaggerate the main conclusions.
- **Keywords:** Three to ten pertinent keywords need to be added after the abstract. We recommend that the keywords are specific to the article, yet reasonably common within the subject discipline.

### Research Manuscript Sections

- **Introduction:** The introduction should briefly place the study in a broad context and highlight why it is important. It should define the purpose of the work and its significance, including specific hypotheses being tested. The current state of the research field should be reviewed carefully and key publications cited. Please highlight controversial and diverging hypotheses when necessary. Finally, briefly mention the main aim of the work and highlight the main conclusions. Keep the introduction comprehensible to scientists working outside the topic of the paper.
- **Materials and Methods:** They should be described with sufficient detail to allow others to replicate and build on published results. New methods and protocols should be described in detail while well-established methods can be briefly described and appropriately cited. Give the name and version of any software used and make clear whether computer code used is available. Include any pre-registration codes.
- **Results:** Provide a concise and precise description of the experimental results, their interpretation as well as the experimental conclusions that can be drawn.
- **Discussion:** Authors should discuss the results and how they can be interpreted in perspective of previous studies and of the working hypotheses. The findings and their implications should be discussed in the broadest context possible and limitations of the work highlighted. Future research directions may also be mentioned. This section may be combined with Results.
- **Conclusions:** This section is not mandatory, but can be added to the manuscript if the discussion is unusually long or complex.
- **Patents:** This section is not mandatory, but may be added if there are patents resulting from the work reported in this manuscript.

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### Back Matter

- **Supplementary Materials:** Describe any supplementary material published online alongside the manuscript (figure, tables, video, spreadsheets, etc.). Please indicate the name and title of each element as follows Figure S1: title, Table S1: title, etc.
- **Acknowledgments:** All sources of funding of the study should be disclosed. Clearly indicate grants that you have received in support of your research work and if you received funds to cover publication costs. Note that some funders will not refund article processing charges (APC) if the funder and grant number are not clearly and correctly identified in the paper. Funding information can be entered separately into the submission system by the authors during submission of their manuscript. Such funding information, if available, will be deposited to FundRef (http://www.crossref.org/fundref/) if the manuscript is finally published.
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  For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, X.X. and Y.Y.; Methodology, X.X.; Software, X.X.; Validation, X.X., Y.Y. and Z.Z.; Formal Analysis, X.X.; Investigation, X.X.; Resources, X.X.; Data Curation, X.X.; Writing – Original Draft Preparation, X.X.; Writing – Review & Editing, X.X.; Visualization, X.X.; Supervision, X.X.; Project Administration, X.X.; Funding Acquisition, Y.Y.", please turn to the CRediT taxonomy (https://www.mdpi.com/data/contributor-role-instruction.pdf) for the term explanation. For more background on CRediT, see here (http://docs.casrai.org/CRediT). "**Authorship must include and be limited to those who have contributed substantially to the work. Please read the section concerning the criteria to qualify for authorship carefully**".
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In the text, reference numbers should be placed in square brackets [ ], and placed before the punctuation; for example [1], [1–3] or [1,3]. For embedded citations in the text with pagination, use both parentheses and brackets to indicate the reference number and page numbers; for example [5] (p. 10). or [6] (pp. 101–105).

The reference list should include the full title, as recommended by the ACS style guide. Style files for Endnote (http://endnote.com/downloads/style/mdpi) and Zotero (https://www.zotero.org/styles/?q=id%3Amultidisciplinary-digital-publishing-institute) are available.

References should be described as follows, depending on the type of work:

- Journal Articles:

1. Author 1, A.B.; Author 2, C.D. Title of the article. *Abbreviated Journal Name* **Year**, *Volume*, page range. Available online: URL (accessed on Day Month Year).

- Books and Book Chapters:

2. Author 1, A.; Author 2, B. *Book Title*, 3rd ed.; Publisher: Publisher Location, Country, Year; pp. 154–196.
3. Author 1, A.; Author 2, B. Title of the chapter. In *Book Title*, 2nd ed.; Editor 1, A., Editor 2, B., Eds.; Publisher: Publisher Location, Country, Year; Volume 3, pp. 154–196.

- Unpublished work, submitted work, personal communication:

4. Author 1, A.B.; Author 2, C. Title of Unpublished Work. status (unpublished; manuscript in preparation).
5. Author 1, A.B.; Author 2, C. Title of Unpublished Work. *Abbreviated Journal Name* stage of publication (under review; accepted; in press).
6. Author 1, A.B. (University, City, State, Country); Author 2, C. (Institute, City, State, Country). Personal communication, Year.

- Conference Proceedings:

7. Author 1, A.B.; Author 2, C.D.; Author 3, E.F. Title of Presentation. In *Title of the Collected Work* (if available), Proceedings of the Name of the Conference, Location of Conference, Country, Date of Conference; Editor 1, Editor 2, Eds. (if available); Publisher: City, Country, Year (if available); Abstract Number (optional), Pagination (optional).

- Thesis:

8. Author 1, A.B. Title of Thesis. Level of Thesis, Degree-Granting University, Location of University, Date of Completion.

- Websites:

9. Title of Site. Available online: URL (accessed on Day Month Year).

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See the Reference List and Citations Guide (https://www.mdpi.com/authors/references) for more detailed information.

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## Preparing Figures, Schemes and Tables

- File for Figures and schemes must be provided during submission in a single zip archive and at a sufficiently high resolution (minimum 1000 pixels width/height, or a resolution of 300 dpi or higher). Common formats are accepted, however, TIFF, JPEG, EPS and PDF are preferred.
- *Nutrients* can publish multimedia files in articles or as supplementary materials. Please contact the editorial office for further information.
- All Figures, Schemes and Tables should be inserted into the main text close to their first citation and must be numbered following their number of appearance (Figure 1, Scheme I, Figure 2, Scheme II, Table 1, *etc.*).
- All Figures, Schemes and Tables should have a short explanatory title and caption.

- All table columns should have an explanatory heading. To facilitate the copy-editing of larger tables, smaller fonts may be used, but no less than 8 pt. in size. Authors should use the Table option of Microsoft Word to create tables.
- Authors are encouraged to prepare figures and schemes in color (RGB at 8-bit per channel). There is no additional cost for publishing full color graphics.

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## Supplementary Materials, Data Deposit and Software Source Code

*Data Availability*

In order to maintain the integrity, transparency and reproducibility of research records, authors must make their experimental and research data openly available either by depositing into data repositories or by publishing the data and files as supplementary information in this journal.

*Computer Code and Software*

For work where novel computer code was developed, authors should release the code either by depositing in a recognized, public repository or uploading as supplementary information to the publication. The name and version of all software used should be clearly indicated.

*Supplementary Material*

Additional data and files can be uploaded as "Supplementary Files" during the manuscript submission process. The supplementary files will also be available to the referees as part of the peer-review process. Any file format is acceptable, however we recommend that common, non-proprietary formats are used where possible.

*Unpublished Data*

Restrictions on data availability should be noted during submission and in the manuscript. "Data not shown" should be avoided: authors are encouraged to publish all observations related to the submitted manuscript as Supplementary Material. "Unpublished data" intended for publication in a manuscript that is either planned, "in preparation" or "submitted" but not yet accepted, should be cited in the text and a reference should be added in the References section. "Personal Communication" should also be cited in the text and reference added in the References section. (see also the MDPI reference list and citations style guide).

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Data may be deposited with specialized service providers or institutional/subject repositories, preferably those that use the DataCite mechanism. Large data sets and files greater than 60 MB must be deposited in this way. For a list of other repositories specialized in scientific and experimental data, please consult databib.org or re3data.org. The data repository name, link to the data set (URL) and accession number, doi or handle number of the data set must be provided in the paper. The journal Data (https://www.mdpi.com/journal/data) also accepts submissions of data set papers.

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New sequence information must be deposited to the appropriate database prior to submission of the manuscript. Accession numbers provided by the database should be included in the submitted manuscript. Manuscripts will not be published until the accession number is provided.

- *New nucleic acid sequences* must be deposited in one of the following databases: GenBank, EMBL, or DDBJ. Sequences should be submitted to only one database.
- *New high throughput sequencing (HTS) datasets* (RNA-seq, ChIP-Seq, degradome analysis, …) must be deposited either in the GEO database or in the NCBI's Sequence Read Archive.
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- *New protein sequences* obtained by protein sequencing must be submitted to UniProt (submission tool SPIN).

All sequence names and the accession numbers provided by the databases should be provided in the Materials and Methods section of the article.

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## Research and Publication Ethics

### Research Ethics

### Research Involving Human Subjects

When reporting on research that involves human subjects, human material, human tissues, or human data, authors must declare that the investigations were carried out following the rules of the Declaration of Helsinki of 1975 (https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki/ (https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki//)), revised in 2013. According to point 23 of this declaration, an approval from an ethics committee should have been obtained before undertaking the research. At a minimum, a statement including the project identification code, date of approval and name of the ethics committee or institutional review board should be cited in the Methods Section of the article. Data relating to individual participants must be described in detail, but private information identifying participants need not be included unless the identifiable materials are of relevance to the research (for example, photographs of participants' faces that show a particular symptom). Editors reserve the right to reject any submission that does not meet these requirements.

Example of an ethical statement: "All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of XXX (Project identification code)."

A written informed consent for publication must be obtained from participating patients who can be identified (including by the patients themselves). Patients' initials or other personal identifiers must not appear in any images. For manuscripts that include any case details, personal information, and/or images of patients, authors must obtain

signed informed consent from patients (or their relatives/guardians) before submitting to an MDPI journal. Patient details must be anonymized as far as possible, e.g., do not mention specific age, ethnicity, or occupation where they are not relevant to the conclusions.

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The editors will require that the benefits potentially derived from any research causing harm to animals are significant in relation to any cost endured by animals, and that procedures followed are unlikely to cause offense to the majority of readers. Authors should particularly ensure that their research complies with the commonly-accepted '3Rs':

- Replacement of animals by alternatives wherever possible,
- Reduction in number of animals used, and
- Refinement of experimental conditions and procedures to minimize the harm to animals.

Any experimental work must also have been conducted in accordance with relevant national legislation on the use of animals for research. For further guidance authors should refer to the Code of Practice for the Housing and Care of Animals Used in Scientific Procedures [1].

Manuscripts containing original descriptions of research conducted in experimental animals must contain details of approval by a properly constituted research ethics committee. As a minimum, the project identification code, date of approval and name of the ethics committee or institutional review board should be cited in the Methods section.

*Nutrients* endorses the ARRIVE guidelines (www.nc3rs.org.uk/ARRIVE (http://www.nc3rs.org.uk/ARRIVE)) for reporting experiments using live animals. Authors and reviewers can use the ARRIVE guidelines as a checklist, which can be found at www.nc3rs.org.uk/ARRIVEchecklist (http://www.nc3rs.org.uk/ARRIVEchecklist).

1. Home Office. Animals (Scientific Procedures) Act 1986. Code of Practice for the Housing and Care of Animals Used in Scientific Procedures. Available online: http://www.official-documents.gov.uk/document/hc8889/hc01/0107/0107.pdf (http://www.official-documents.gov.uk/document/hc8889/hc01/0107/0107.pdf).

### Research Involving Cell Lines

Methods sections for submissions reporting on research with cell lines should state the origin of any cell lines. For established cell lines the provenance should be stated and references must also be given to either a published paper or to a commercial source. If previously unpublished *de novo* cell lines were used, including those gifted from another

laboratory, details of institutional review board or ethics committee approval must be given, and confirmation of written informed consent must be provided if the line is of human origin.

An example of Ethical Statements:

The HCT116 cell line was obtained from XXXX. The MLH1$^{+}$ cell line was provided by XXXXX, Ltd. The DLD-1 cell line was obtained from Dr. XXXX. The DR-GFP and SA-GFP reporter plasmids were obtained from Dr. XXX and the Rad51K133A expression vector was obtained from Dr. XXXX.

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The editors of this journal enforce a rigorous peer-review process together with strict ethical policies and standards to ensure to add high quality scientific works to the field of scholarly publication. Unfortunately, cases of plagiarism, data falsification, image manipulation, inappropriate authorship credit, and the like, do arise. The editors of *Nutrients* take such publishing ethics issues very seriously and are trained to proceed in such cases with a zero tolerance policy.

Authors wishing to publish their papers in *Nutrients* must abide to the following:

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- Authors should accurately present their research findings and include an objective discussion of the significance of their findings.
- Data and methods used in the research need to be presented in sufficient detail in the paper, so that other researchers can replicate the work.
- Raw data should preferably be publicly deposited by the authors before submission of their manuscript. Authors need to at least have the raw data readily available for presentation to the referees and the editors of the journal, if requested. Authors need to ensure appropriate measures are taken so that raw data is retained in full for a reasonable time after publication.
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During the submission process, please suggest three potential reviewers with the appropriate expertise to review the manuscript. The editors will not necessarily approach these referees. Please provide detailed contact information (address, homepage, phone, e-mail address). The proposed referees should neither be current collaborators of the co-authors nor have published with any of the co-authors of the manuscript within the last five years. Proposed reviewers should be from different institutions to the authors. You may identify appropriate Editorial Board members of the journal as potential reviewers. You may suggest reviewers from among the authors that you frequently cite in your paper.

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## English Corrections

To facilitate proper peer-reviewing of your manuscript, it is essential that it is submitted in grammatically correct English. Submitted manuscripts that fail to fulfil this requirement will usually be rejected. Advice on some specific language points can be found here (https://www.mdpi.com/authors/english-editing).

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Expanded and high quality conference papers can be considered as articles if they fulfil the following requirements: (1) the paper should be expanded to the size of a research article; (2) the conference paper should be cited and noted on the first page of the paper; (3) if the authors do not hold the copyright of the published conference paper, authors should seek the appropriate permission from the copyright holder; (4) authors are asked to disclose that it is conference paper in their cover letter and include a statement on what has been changed compared to the original conference paper. *Nutrients* does not publish pilot studies or studies with inadequate statistical power.

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## Qualification for Authorship

Each author is expected to have made substantial contributions to the conception or design of the work; acquisition, analysis, or interpretation of data; the creation of new software used in the work; and/or writing or substantively revising the manuscript. In addition, all authors must have approved the submitted version (and any substantially modified version that involves the author's contribution to the study); AND agrees to be personally accountable for the author's own contributions and for ensuring that questions related to the accuracy or integrity of any part of the work, even those in which the author was not personally involved, are appropriately investigated, resolved, and documented in the literature. Note that acquisition of funding, collection of data, or general supervision of the research group do not, by themselves, justify authorship.Those who contributed to the work but do not qualify for authorship should be listed in the acknowledgements.

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and the other authors and should keep co-authors informed and involve them in major decisions about the publication (e.g. answering reviewers' comments)." [1]. We reserve the right to request confirmation that all authors meet the authorship conditions.

1. Wager, E.; Kleinert, S. Responsible research publication: international standards for authors. A position statement developed at the 2nd World Conference on Research Integrity, Singapore, July 22-24, 2010. In Promoting Research Integrity in a Global Environment; Mayer, T., Steneck, N., eds.; Imperial College Press / World Scientific Publishing: Singapore; Chapter 50, pp. 309-16.

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