UNIVERSIDADE ESTADUAL DE MONTES CLAROS

Letícia Antunes Athayde

Avaliação experimental dos efeitos moduladores do tratamento oral com *Lactococcus lactis* sobre a inflamação alérgica e alteração da microbiota intestinal desencadeados pela ingestão aguda de álcool

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Tese apresentada ao Programa de Pós-graduação em Ciências em Saúde da Universidade Estadual de Montes Claros-Unimontes, como parte das exigências para a obtenção do título de Doutora em Ciências da Saúde.

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Orientadores: Profa. Dra. Mariléia Chaves Andrade

Prof. Dr. Sergio Nobre Avelino Mota Nobre

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RESUMO

O consumo de álcool é capaz de desencadear efeitos inflamatórios no trato gastrointestinal interferindo com a homeostasia da mucosa e induzindo uma resposta inflamatória do tipo alérgica, apresentando níveis de IgE séricos elevados e níveis aumentados de interleucina (IL)-4 na mucosa gástrica. Vários estudos têm demonstrado que o consumo excessivo de álcool altera a composição da microbiota intestinal em modelos de roedores e em seres humanos, causando perturbação da homeostase da microbiota. O objetivo deste estudo foi avaliar os efeitos moduladores do tratamento oral com *Lactococcus lactis* sobre a inflamação alérgica e alteração da microbiota intestinal desencadeados pela ingestão aguda de álcool em camundongos. Durante quatro dias consecutivos, foi realizada a administração intragástrica de 0,2 mL de etanol 50% por animal ou solução salina, e vinte e quatro horas após a última administração, os animais receberam, em mamadeiras, *Lactococcus lactis*, caldo M17 ou somente água, durante dois dias consecutivos. Logo após o término do segundo dia do tratamento *ad libitum*, os animais foram sacrificados, e realizadas as coletas de sangue, estômago e intestino delgado, para análises imunológicas e histológicas, e coleta de estômago, intestino delgado e intestino grosso (cólon), para análises microbiológicas. Em relação às análises imunológicas e morfofuncionais, o tratamento com *Lactococcus lactis* foi capaz de restaurar a níveis basais a IgA secretória na mucosa gástrica, IgE total sérica, a produção de IL-4 nas mucosas gástrica e intestinal e níveis de IL-10 na mucosa gástrica. Além disso, *Lactococcus lactis* reduziu a degeneração hepática provocada pelo etanol, os níveis de IL-10 na mucosa intestinal, e aumentou os níveis de IFN-γ na mucosa gástrica. Quanto as análises microbiológicas, a ingestão de etanol pelos animais provocou um desprendimento mais acentuado das *Enterobacteriaceae* da mucosa do estômago e intestino delgado e reduziu as populações de BAL presuntivas e de *L. lactis* presuntivo em todo TGI. O tratamento com *L. lactis* estimulou a diversificação das populações de *Enterobacteriaceae* em todo TGI, principalmente de espécies comensais. O presente estudo abre perspectivas para a utilização terapêutica de *Lactococcus lactis* no tratamento de processos inflamatórios alérgicos desencadeados pela ingestão aguda de álcool e na modulação bioterapêutica da microbiota intestinal, podendo ser uma estratégia promissora para reduzir as injurias induzidas pelo álcool.

Palavras-chave: Etanol. *Lactococcus lactis*. Inflamação alérgica. Microbiota intestinal.

ABSTRACT

The alcohol can trigger inflammatory effects in the gastrointestinal tract interfering with mucosal homeostasis and inducing an inflammatory response of allergic type, having high serum immunoglobulin (Ig) E levels and increased levels of interleukin (IL)-4 gastric mucosa. Several studies have shown that excessive alcohol consumption alters the composition of intestinal microbiota in rodents and humans, disrupting the microbiota homeostasis. The aim of this study was to evaluate the modulatory effects of oral treatment with *Lactococcus lactis* on allergic inflammation and altered intestinal microbiota triggered by acute alcohol intake in mice. For four consecutive days, intragastric administration of 0.2 mL of 50% ethanol per animal or saline solution, and twenty-four hours after the last administration, the animals were given in bottles, *Lactococcus lactis*, M17 broth or water only for two consecutive days. The completion of the second day of *ad libitum* treatment, the animals were sacrificed, and made collections of blood, stomach and small intestine, to immunological and histological analysis, and collection of the stomach, small intestine and large intestine (colon), for microbiological analysis. Regarding immunological, morphological and functional analyzes, treatment with *Lactococcus lactis* was able to restore basal levels of secretory IgA in the gastric mucosa, serum total IgE, IL-4 production in gastric and intestinal mucosa and IL-10 levels in the gastric mucosa. Moreover, *Lactococcus lactis* reduced hepatic degeneration caused by ethanol, IL-10 levels in the intestinal mucosa, and increased IFN-g levels in the gastric mucosa. The microbiological analysis, ingestion of ethanol by animals strongly detached *Enterobacteriaceae* from the stomach mucosa and small intestine and reduced presumptive lactic acid bacteria and presumptive *L. lactis* populations in the GIT. *L. lactis* treatment encouraged the diversification of *Enterobacteriaceae* population, particularly the commensal species, in the GIT. This study opens perspectives for the therapeutic use of *Lactococcus lactis* in the treatment of allergic inflammatory processes triggered by acute ingestion of alcohol and biotherapeutic modulation of the intestinal microbiota with this LAB appears to be a promising strategy to reduce alcohol-induced injuries.

Keywords: Ethanol. *Lactococcus lactis*. Allergic inflammation. Intestinal microbiota.

LISTA DE ABREVIATURAS E SIGLAS

APCs	Células apresentadoras de antígeno
BAL	Bactérias ácido lácticas
FDA	*Food and Drug Administration*
GALT	Tecido linfoide associado ao trato gastrointestinal
GRAS	*Generally Recognized as Safe*
IgAs	Imunoglobulina do tipo A secretória
IgE	Imunoglobulina do tipo E
IFN-γ	Interferon-gama
IL-2	Interleucina-2
IL-4	Interleucina-4
IL-10	Interleucina-10
L. lactis	*Lactococcus lactis*
LAP	*Latency Associated Peptide*
LPS	Lipopolissacarídeo
MALT	Tecido linfoide associado a mucosas
NK	*Natural-killer*
pH	Potencial hidrogeniônico
TGF-β	Fator de transformação do crescimento-beta
TGI	Trato gastrointestinal
TNF-α	Fator de necrose tumoral

APRESENTAÇÃO

Esta tese segue a formatação preconizada pelo PPGCS - Unimontes, onde dispõe de uma primeira seção com a introdução e os objetivos do trabalho.

Uma segunda seção apresenta os produtos (artigos redigidos seguindo normas do periódico escolhido, incluindo lista de referências utilizadas especificamente em cada artigo). No caso de tese de doutorado, o PPGCS exige apresentação de, no mínimo, dois artigos.

A terceira seção é composta pelas considerações finais e/ou conclusões, referências das citações utilizadas na introdução.

Anexos e Apêndices estão incluídos após as referências, nos trabalhos em que houver necessidade de apresentar documentação complementar e/ou comprobatória. Ressalta-se que as normas da revista devem ser apresentadas em anexo. Maiores detalhes sobre a formatação e normatização adotadas pelo PPGCS podem ser obtidos no endereço eletrônico <www.ppgcs.unimontes.br>.

SUMÁRIO

1 INTRODUÇÃO

Vários eventos ocorrem no trato gastrointestinal (TGI) de mamíferos, tais como a identificação, digestão, modificação e a absorção de componentes alimentares da dieta (proteínas, lipídios, carboidratos e micronutrientes, como vitaminas e minerais). Além disso, é nele que ocorre a interação de microrganismos, tanto patogênicos como não patogênicos. As interações com antígenos da dieta e da microbiota desencadeiam eventos imunológicos que se caracterizam por respostas reguladoras locais e sistêmicas (1-3).

Para que tudo ocorra com segurança, a mucosa intestinal é composta de uma camada simples de células epiteliais intestinais e da lâmina própria. O epitélio separa o ambiente externo do interno e assim atua prevenindo que microrganismos patogênicos, bem como substâncias nocivas no lúmen, ganhem acesso ao corpo. O epitélio intestinal é formado principalmente por enterócitos com junções celulares e borda em escova, seguido de outros tipos celulares como os linfócitos intraepiteliais, as células M, as células de *Paneth* e as células caliciformes (4). O epitélio intestinal, o muco (produzido pelas células caliciformes), o potencial hidrogeniônico (pH) ácido gástrico, as enzimas luminais, os sais biliares, a microbiota residente e a motilidade intestinal, fazem parte de um conjunto de elementos que juntos formam os mecanismos fisiológicos do TGI (2, 5, 6).

Além do epitélio da mucosa intestinal, o TGI também é composto por um tecido linfoide associado a mucosas (MALT), e que por sua localização, esse tecido é denominado tecido linfoide associado ao trato gastrointestinal (GALT). O GALT é formado por ambos os sistemas imunes: o inato (células *natural-killer* [NK], leucócitos polimorfonucleados, macrófagos, células epiteliais e receptores *toll-like*) e o adaptativo (linfócitos intraepiteliais e da lâmina própria, placas de Peyer, imunoglobulinas do tipo A secretória [IgAs] e citocinas), formando os mecanismos imunológicos do TGI (2, 6-9).

O TGI humano é colonizado por um grande número de microrganismos que habitam o trato intestinal e desempenham uma variedade de funções fisiológicas. Esta microbiota fornece não só uma importante barreira entre o hospedeiro e o ambiente, mas também sítios de contato entre os microrganismos e o sistema imune em desenvolvimento (10). O TGI comporta uma microbiota complexa de mais de 500 espécies bacterianas diferentes. Uma estimativa mostra

que existem 10 vezes mais células bacterianas que células eucarióticas no corpo. O trato gastrointestinal é estéril ao nascimento, mas é colonizado por microrganismos presentes no meio ambiente imediatamente após o parto. Inicialmente, quando o espaço e a disponibilidade de alimentos são abundantes, as bactérias com altas taxas de multiplicação começam a dominar. A partir do momento que o número bacteriano aumenta, a disponibilidade de alimento e espaço diminui e o hábitat fica ocupado por microrganismos mais especializados e a complexidade da microbiota aumenta (11).

Uma vez estabelecida, a microbiota intestinal permanece relativamente estável, embora sofra variações quantitativas e qualitativas, dependendo da espécie animal a qual está associada, bem como de sua localização orgânica na espécie humana. A cavidade oral aloja mais de 200 espécies de microrganismos. O estômago e os dois terços do intestino delgado proximal (duodeno e jejuno) contêm um número pequeno de microrganismos (10^3-10^4 bactérias/mL de conteúdo gástrico ou intestinal). Isto se deve ao baixo pH do estômago e a constante mobilidade (peristaltismo) nessas áreas. Em ratos, por exemplo, onde o pH estomacal é relativamente alto, a densidade microbiana aumenta (10^5-10^6 bactérias/mL de conteúdo gástrico). Os principais tipos de microrganismos encontrados são lactobacilos e estreptococos, que ao contrário da maioria dos microrganismos encontrados nos alimentos, sobrevivem à passagem pelo estômago. A parte distal do intestino delgado é considerada "zona de transição" entre o baixo nível populacional do intestino delgado proximal e o enorme número de bactérias encontradas no intestino grosso. Tanto em roedores como em humanos constata-se um aumento progressivo dos níveis populacionais ao longo do intestino delgado. O intestino grosso (cólon) é a região mais densamente colonizada em animais e humanos, provavelmente devido à sua baixa motilidade e baixo potencial de oxirredução. Logo, o cólon comporta um grande número de microrganismos, dentre 400 e 500 espécies (12).

As comunidades microbianas comensais do intestino apresentam alta diversidade no nível de espécie, porém baixa diversidade em nível de filo. Em todos os vertebrados, a microbiota comensal intestinal é dominada por dois filos: os Gram negativos *Bacteroidetes* e os Gram positivos *Firmicutes*, compreendendo cerca de 90% dos filos presentes no intestino. Os outros 10% da população total pertencem predominantemente aos filos *Proteobacteria* e *Actinobacteria* (13).

As bactérias pertencentes ao filo *Proteobacteria* são normalmente detectados nas amostras gastrointestinais e este grupo de bactérias Gram-negativas é particularmente diversificado, embora não muito abundante, cerca de 1% da microbiota total (14). Existem cinco classes diferentes de *Proteobacteria*, tais como, o alfa-, beta-, gama-, delta e epsilonproteobacteria no qual as *Enterobacteriaceae* são as mais abundantes e predominantes. A maioria dos membros de *Enterobacteriaceae* está associada com diarreia (15), embora os representantes desta família não são necessariamente causadores de quaisquer sintomas e são, na verdade, um dos primeiros a serem encontrados no trato gastrointestinal do recém-nascido (16). A *Escherichia coli* é o representante mais prevalente desta família sendo muitas vezes a bactéria anaeróbia facultativa mais abundante nas amostras gastrointestinais. As diferentes cepas de *Escherichia coli* podem apresentar propriedades diferentes, variando de probiótico (17) para patogênicos, causando diarreia ou infecções em outros locais (18). A maioria das outras *Enterobacteriaceae* spp. raramente são isoladas a partir de amostras gastrointestinais (14).

O fator predominante para a colonização das bactérias no trato gastrointestinal é a sua capacidade de adesão aos receptores da mucosa intestinal, sendo possível que microrganismos pioneiros exerçam um papel fundamental na seleção da microbiota, propiciando um ambiente favorável para eles, impedindo o crescimento de outros microrganismos (19). Depois de estarem aderidos à mucosa intestinal, os microrganismos estabelecem colônias permanentes, constituindo a microbiota autóctone que, com o amadurecimento da relação simbiótica com o hospedeiro torna-se cada vez mais estável. Outros microrganismos, introduzidos posteriormente, podem se associar à mucosa, porém sem adesão a receptores, constituindo a microbiota alóctone (20).

O sistema imune tem se desenvolvido e evoluído de uma maneira bem eficiente para controlar e viver com a população de microrganismos com o qual está associado. Para que exista essa relação, o hospedeiro se protege contra invasões microbianas, lesões e também a reações indesejadas contra antígenos presentes nos alimentos, enquanto os microrganismos intestinais necessitam de proteção contra microrganismos competitivos e contra a própria resposta imune do hospedeiro (21). O epitélio participa ativamente no processo de reconhecimento da microbiota. Ele funciona como uma barreira física, impedindo a invasão dos microrganismos mantendo a microbiota no lúmen intestinal, e produzindo substâncias (peptídeos antimicrobianos, defensinas, IgA, muco) que inibem o crescimento excessivo de bactérias indesejáveis (22). Além disso, as bactérias da microbiota limitam adesão e crescimento de

bactérias anaeróbicas gram-negativas patogênicas, também naturalmente presentes no intestino humano. Alteração desse equilíbrio permite crescimento e adesão das bactérias patogênicas e, possivelmente, a translocação bacteriana (23, 24).

As bactérias nativas influenciam no desenvolvimento dos componentes humorais do sistema imunológico e modulam o perfil de citocinas Th1 e Th2 (25, 26). Normalmente as bactérias são mortas pelo sistema retículoendotelial *in situ* ou quando estão a caminho dos órgãos linfóides. Sendo assim, o linfonodo mesentérico e outros sítios extra intestinais permanecem sem bactérias (23).

As citocinas do tipo Th1, como o interferon-gama (IFN-γ) e fator de necrose tumoral (TNF-α), aumentam a imunidade mediada por células. O efeito Th1 predominante resulta na ativação de macrófagos e linfócitos T, particularmente os citotóxicos. As citocinas Th2, incluindo a interleucina-4 (IL-4) e IL-10, aumentam a imunidade humoral, resultando em ativação de linfócitos B e aumento da produção de anticorpos. Os efeitos Th1 e Th2 são contrarregulatórios, por isso, no indivíduo saudável, a mucosa intestinal se encontra em estado inflamatório controlado (27, 28).

Interações imunológicas-microbianas bidirecionais regulam o desenvolvimento da imunidade das mucosas e alteram a composição da microbiota, contribuindo para o bem-estar geral do hospedeiro. O sistema imunológico da mucosa de crianças amadurece ao longo de vários meses após o nascimento, estando este processo intimamente ligado com o desenvolvimento e estabelecimento da microbiota intestinal, além da qualidade dos nutrientes da dieta e metabólitos produzidos pelos comensais (29). Exemplos desses metabólitos são os ácidos graxos de cadeia curta que são capazes de se ligar a receptores presentes na superfície de células imunes, como o receptor acoplado a proteína G (GPR43), modulando a resposta inflamatória nessas células (30). Dessa forma, a microbiota possui um papel importante na maturação e homeostase do sistema imune.

Desordens gastrointestinais podem ocorrer quando os mecanismos de defesa da mucosa são quebrados por agentes infecciosos e irritantes, doenças autoimunes, fumo, estresse, uso prolongado de anti-inflamatórios não esteroidais e ingestão de álcool (31, 32).

O álcool é uma droga lícita altamente consumida nas sociedades ocidentais, o que poderia potencialmente impactar na comunidade da microbiota intestinal. Vários estudos têm demonstrado que o consumo excessivo de álcool altera a composição da microbiota intestinal em modelos de roedores e em seres humanos, causando perturbação da homeostase da microbiota, denominada de disbiose (33-36).

A maioria dos estudos indicam uma associação entre crescimento bacteriano intestinal induzido pelo álcool e disbiose, e o desenvolvimento/progressão da doença hepática alcoólica e cirrose (37). Estudos mostram que o consumo de álcool rompe a barreira intestinal (38) por meio de aumento da carga de *stress* oxidativo no intestino, o que por sua vez perturba as "*tight junction*" e promove a hiperpermeabilidade intestinal (39). O aumento da permeabilidade intestinal permite a translocação de produtos microbianos pró-inflamatórios/patogênicos, incluindo as endotoxinas (por exemplo, lipopolissacarídeo [LPS] e peptidoglicano), a partir do lúmen intestinal para o fígado através da veia porta (40). A exposição a estes produtos bacterianos provoca inflamação no fígado que em associação com os efeitos diretos do álcool pode causar a doença hepática alcoólica (41).

Além do efeito do álcool na microbiota intestinal, estudos das duas últimas décadas têm mostrado que o consumo de álcool resulta em alterações de componentes celulares do sistema imune inato e adaptativo (42). O abuso do álcool é considerado como sendo um fator que interfere na atividade imunológica, causando atrofia do baço e do timo, além de um impacto na redistribuição de leucócitos do sangue periférico devido a uma diminuição da habilidade de migração de leucócitos após injúria ou infecção. Além disso, provoca anormalidades funcionais em células NK e em linfócitos T e B, causando uma diminuição das respostas imune celular e humoral (43).

Dentre as principais alterações no sistema imune, podem-se citar aquelas observadas nas células apresentadoras de antígeno (APCs), que têm seus aspectos fenotípicos e funcionais modificados pela exposição ao álcool. As APCs são componentes especializados do sistema imune inato, que possuem um importante papel na ativação da resposta imune adaptativa, visto que apresentam o antígeno para os linfócitos T, levando à ativação dessa população celular e desencadeando o início de uma resposta imune específica efetiva (44, 45).

Em estudo utilizando o modelo de administração intragástrica aguda de etanol (46), foi identificado um impacto diferencial do álcool sobre aspectos imunofenotípicos, síntese de citocinas, e capacidade fagocítica de subpopulações de células apresentadoras de antígenos (46, 47). Os efeitos do álcool, nesse modelo, favoreceram o desenvolvimento de uma resposta inflamatória alérgica. Em síntese, os efeitos do etanol sobre as populações de macrófagos e células dendríticas parecem ser moduladores da atividade celular, enquanto sobre os linfócitos B, o efeito seria estimulador, destacado por um aumento da capacidade endocítica e da expressão de moléculas coestimuladoras (47).

Em função desse provável desvio da hierarquia das APCs após a administração oral de etanol, os linfócitos T apresentam-se mais ativados devido a um aumento na expressão de receptores de citocinas mitogênicas, como a IL-2 e IL-4, diminuição da expressão da selectina CD62-L, além da diminuição da expressão da forma latente do TGF-β (Fator de transformação do crescimento – beta) expresso na membrana celular (LAP, *Latency Associated Peptide*) (47). Além disso, foram observadas alterações locais e sistêmicas como níveis aumentados de IL-4 no estômago e IgE total sérica, aumento na síntese de IL-4 e ausência de IL-10 por esplenócitos. Somado a isto, a ingestão de etanol preveniu a tolerância oral induzida por Ovalbumina (46), um fenômeno fisiológico e T - dependente que ocorre na superfície da mucosa intestinal e que mantém a regulação da reatividade imunológica inflamatória a antígenos da dieta e da microbiota autócrina (48).

Esses resultados em conjunto sugerem que alterações imunológicas induzidas pelo álcool interferem com mecanismos imuno-reguladores em toda a mucosa intestinal, possivelmente, com efeitos sistêmicos, levando a um perfil alergênico com consequente perda da susceptibilidade à indução de tolerância oral a uma nova proteína. Além disso, o consumo excessivo de álcool altera a composição da microbiota intestinal causando perturbação da homeostase da microbiota.

Atualmente, os tratamentos para danos gerados pelo álcool se restringem a intervenções medicamentosas direcionadas ao quadro patológico presente e no uso específico de medicamentos voltados para o controle da dependência de álcool, entretanto alguns fármacos aversivos oferecem inúmeros efeitos adversos. Desta forma, a busca de estratégias alternativas capazes de prevenir os efeitos do consumo de álcool a nível local e sistêmico se torna de extrema relevância.

Uma alternativa inovadora de tratamento de doenças que acometem o trato gastrointestinal, ainda em fase experimental, é a administração oral de microrganismos produtores de imunomoduladores. Neste caso, microrganismos que fossem bons produtores de proteínas heterólogas, capazes de resistir às intempéries do trato gastrointestinal, transientes, e pouco imunogênicos, seriam candidatos ideais neste estudo. Baseado nestes requisitos, as bactérias ácido lácticas (BAL) poderiam, perfeitamente, desempenhar tal função.

A designação “bactérias lácticas” se aplica a um grupo de bactérias gram positivas, não patogênicas, que têm o ácido láctico como principal produto metabólico da fermentação de carboidratos. As principais espécies do grupo bactérias lácticas são *Lactobacillus, Leuconostoc, Pediococcus, Streptococcus e Lactococcus* (49-51).

Com poucas exceções, as bactérias lácticas obtêm sua energia a partir da conversão de açúcares, principalmente a glicose, em ácido láctico (via homofermentativa ou homoláctica) e/ou ácido láctico e outros produtos (via heterofermentativa ou mista) (51). Consequentemente, as bactérias lácticas estão geralmente associadas com a preparação de alimentos fermentados, como iogurtes, queijos, leites fermentados, pães, manteiga, vinhos, salsichas, picles e silagem. Este processo, conhecido como "fermentação láctica dos alimentos", remonta a cerca de 8.000 a.C. e constitui uma das formas mais antigas de conservação dos alimentos utilizados pelos seres humanos. A conservação dos alimentos utilizando essas bactérias ocorre não apenas como consequência da acidificação do meio (pH 4,5-3,5), mas também devido à produção de numerosos agentes antibacterianos, tais como bacteriocinas e compostos orgânicos (52). Esses dois fatores inibem o crescimento de uma microbiota indesejável e são responsáveis pelo desenvolvimento de algumas características organolépticas, tais como aroma, textura e sabor do produto final.

Por serem utilizadas há séculos em processos de fermentação e preservação de alimentos, as bactérias ácidos lácticas são diariamente ingeridas por seres humanos sendo consideradas seguras para consumo humano característica que rendeu ao grupo um “status” GRAS (GRAS – do inglês *Generally Recognized As Safe*) de acordo com o *Food and Drug Administration* (FDA), órgão americano que fiscaliza medicamentos e alimentos (52).

Recentemente o potencial para novas aplicações das bactérias lácticas, tais como vacinas orais, produção de proteínas heterólogas e metabólitos vêm sendo explorado por vários grupos de

pesquisa. Estas bactérias têm sido utilizadas como “usinas celulares” para a produção de moléculas de interesse médico e biotecnológico, como citocinas, enzimas, alérgenos e antígenos (53) e também utilizadas como veículo para a apresentação de antígenos exógenos às superfícies de mucosas (49).

Lactococcus lactis (L. lactis) é a espécie de bactérias ácidos lácticas mais bem caracterizada e figura atualmente como organismo modelo no estudo das mesmas; não apenas pela sua importância econômica, mas também devido ao fato de: (i) ser um microrganismo de fácil manipulação; (ii) ser “GRAS”; (iii) ter sido a primeira BAL cujo genoma foi sequenciado (54) e (iv) possuir um grande número de ferramentas genéticas já desenvolvidas (53-56) como, por exemplo, sistemas de mutagênese (57) e vetores de clonagem e expressão gênica (58, 59).

L. lactis é resistente à acidez gástrica quando administrada junto com os alimentos, mantendo-se biologicamente ativa em todo o trajeto através do trato digestivo (60). Além disso, por ser uma bactéria não-invasiva e não-comensal, isto é, de passagem transitória através do intestino dos animais, tem um menor potencial de desencadear imunotolerância ou efeitos colaterais sobre o seu uso prolongado (53). Outra característica importante é que por ser uma bactéria Gram-positiva, *L. lactis* não possui o lipopolissacarídeo (LPS) endotóxico e ainda apresenta menos exoproteínas nativas em comparação com linhagens de *Escherichia coli* (61).

Desta forma, grupos de pesquisa têm explorado o efeito imunomodulador do *Lactococcus lactis* como estratégia protetora da mucosa gastrointestinal dos animais que recebem álcool.

Um estudo recente, avaliando os efeitos do pré-tratamento oral com *L. lactis*, produtor ou não de Hsp65, como estratégia profilática para modulação das alterações inflamatórias desencadeadas pela ingestão aguda de etanol, demonstrou que este pré-tratamento reverteu os sinais clássicos de reação alérgica do tipo I, através da redução dos níveis de IL-4 na mucosa gástrica e intestinal, além dos níveis séricos de IgE total; restaurou a homeostase do muco gástrico secretado e da produção de IL-10 intestinal; impactou diferencialmente no número de APCs ativadas, como macrófagos, células dendríticas e linfócitos B, no linfonodo mesentérico e baço; além disso, *L. lactis* selvagem apresentou uma capacidade em aumentar células T com fenótipo regulador nesses órgãos; e parece resgatar o fenômeno da tolerância oral quebrada pelo consumo de álcool (62).

Pesquisas em roedores e humanos estão investigando se a disbiose intestinal induzida pelo álcool e suas consequências podem ser reversíveis com intervenções com probióticos e simbióticos. Os lactobacilos e as bifidobactérias, atualmente, são os microrganismos mais utilizados como probióticos, e um estudo realizado em camundongos C57BL/6 que ingeriram álcool (5% vol/vol) por via oral durante 6 semanas mostrou que o tratamento oral de *Lactobacillus rhamnosus* GG (1×10^9 UFC/mL), durante 6 a 8 semanas, evitou a disbiose induzida por álcool (33). Entretanto, ainda não foi avaliado o efeito do *Lactococcus lactis* na microbiota intestinal após o consumo de álcool.

Diante disso, o *Lactococcus lactis* surge como nova possibilidade terapêutica no tratamento de alterações imunológicas, com perfil alergênico, e disbiose intestinal desencadeadas pela ingestão aguda de álcool. Este microrganismo emerge como um potente probiótico, capaz de resgatar a homeostase do organismo e estimular mecanismos inatos e adaptativos.

2 OBJETIVOS

2.1 Objetivo geral

- Avaliar os efeitos moduladores do tratamento oral com *Lactococcus lactis* NCDO-2118 sobre a inflamação alérgica e alteração da microbiota intestinal desencadeados pela ingestão aguda de álcool em camundongos.

2.2 Objetivos específicos

- Avaliar o efeito modulador do *Lactococcus lactis* em parâmetros biométricos e morfológicos após administração de etanol.
- Avaliar o efeito modulador do *Lactococcus lactis* na imunidade das mucosas gástrica e intestinal após a administração de etanol.
- Avaliar o efeito do tratamento com *Lactococcus lactis* em biomarcadores relacionados com respostas alérgicas após a ingestão de etanol.
- Avaliar o efeito do tratamento com *Lactococcus lactis* na abundância e diversidade de espécies de *Enterobacteriaceae* no trato gastrointestinal após a administração de etanol.
- Avaliar o efeito do tratamento com *Lactococcus lactis* na abundância de Bactérias ácido lácticas (BAL) presuntivas no trato gastrointestinal após a administração de etanol.
- Avaliar a frequência de isolamento de *Lactococcus lactis* presuntivo no trato gastrointestinal após a ingestão de etanol.

3 PRODUTOS

3.1 Produto 1: Lactococcus lactis *treatment modulates the allergic inflammation induced by acute ethanol ingestion*, formatado segundo as normas para publicação do periódico Scandinavian Journal of Immunology.

3.2 Produto 2: *Effect of* Lactococcus lactis *treatment on* Enterobacteriaceae *and lactic acid bacteria populations in the gastrointestinal tract after ethanol ingestion*, formatado segundo as normas para publicação do periódico Applied and Environmental Microbiology, enviado para este periódico.

Lactococcus lactis treatment modulates the allergic inflammation induced by acute ethanol ingestion

L. lactis modulates the allergic inflammation

Letícia Antunes Athayde, MSc[*], Sarah Leão Fiorini de Aguiar, MSc[†], Mariana Camila Gonçalves Miranda, MSc[†], Ronize Viviane Jorge Brito, MSc[*], Ana Maria Caetano de Faria, PhD [†], Sergio Avelino Mota Nobre, PhD[*], Mariléia Chaves Andrade, PhD[*]

[*]Universidade Estadual de Montes Claros (UNIMONTES), 39401-089, Montes Claros, MG, Brazil;

[†]Universidade Federal de Minas Gerais (UFMG), ICB, UFMG, Belo Horizonte, MG, Brazil.

Corresponding author: Mariléia Chaves Andrade, PhD

Hospital Universitário Clemente de Faria - Av. Cula Mangabeira, 562 - Santo Expedito - 39401-001, Montes Claros-MG, Brazil. E-mail: andrade.marileia@gmail.com

Tel: +55 38 3224-8376

Fax: +55 38 3224-8372

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Abstract

The alcohol can trigger inflammatory effects in the gastrointestinal tract interfering with mucosal homeostasis and inducing an inflammatory response of allergic type, having high serum immunoglobulin (Ig) E levels and increased levels of interleukin (IL)-4 gastric mucosa. The aim of this study was to evaluate the modulatory effects of *Lactococcus lactis* oral treatment on allergic inflammation triggered by acute alcohol intake in mice. C57BL/6 females mice received saline or ethanol intragastrically for 4 consecutive days and 24 hours after the last administration, the animals were *Lactococcus lactis* NCDO-2118, M17 broth or water orally *ad libitum* for two consecutive days. Soon after the second day of treatment, the animals were sacrificed and made the collection of blood, stomach and small intestine, histological and immunological analysis. *Lactococcus lactis* treatment was able to restore basal levels of secretory IgA in the gastric mucosa, serum total IgE, IL-4 production in gastric and intestinal tissues, and IL-10 levels in gastric tissue. Moreover, *Lactococcus lactis* reduced hepatic degeneration caused by ethanol IL-10 levels in the intestinal tissue and increased interferon gamma (IFN-γ) levels in gastric tissue. Thus, this study opens perspectives for the therapeutic use of *Lactococcus lactis* for the treatment of allergic inflammatory processes elicited by acute alcohol ingestion.

Keywords: Ethanol. *Lactococcus lactis.* Allergic inflammation.

Introduction

Alcohol has been the most common substance use and abuse in human history. Moderate amounts of alcohol are appreciated for their anxiolytic effects; however, its addictive properties can lead to chronic, excessive alcohol use disorders and alcohol use. In addition to its behavioral effects generally recognized, alcohol affects many organs, including the immune system that controls the body's defense against infectious agents and other harmful agents [1] .

It is well known that alcohol can trigger inflammatory effects in the gastrointestinal tract interfering with mucosal homeostasis [2, 3] . Previous studies in our group have shown that the high dose of ethanol (EtOH), even for a short time period (four consecutive days), is able to induce an inflammatory response of allergic type, with elevated serum IgE levels, plus increased levels of interleukin (IL)-4 gastric mucosa [2, 4] , and increased IL-4 synthesis by splenic T cells after *in vitro* stimulation nonspecific (concanavalin a - Con a) [4] . These results support the evidence of a strong allergic profile induced by ethanol.

Currently, treatments for damage caused by alcohol are restricted to psychotherapeutic and psychopharmacological interventions. Since the alcohol can cause a variety of diseases associated with consumption, often the treatment of these patients is based on pharmacological interventions directed to this pathological condition, and the particular use of drugs directed to the control of alcohol dependence. Some aversive drugs are widely used, however they offer numerous adverse effects such as nausea, vomiting, abdominal pain, headache, confusion, drowsiness, changes in libido and dermatologic effects. In this context, the use of safe alternative therapies, able to prevent the effects of alcohol consumption at local and systemic level emerges as a strong candidate for the treatment of changes resulting from consumption.

An innovative alternative is the use of probiotics which are live microorganisms administered in adequate amounts, confer a health benefit on the host [5] . Probiotics modulating properties with the composition of the intestinal microbiota has been proposed as tools for the prevention or treatment of alcoholic liver disease [6, 7] .

Most probiotics used and currently studied belong to the group of lactic acid bacteria, particularly lactobacilli, which were isolated from the gastrointestinal tract human, but also includes some *Bifidobacterium* [8] and *Streptococcus* strains [9] . Members of the genus *Lactobacillus* have shown therapeutic properties, the improvement of the normal microbiota

[10] , the prevention of infectious diseases and food allergies [11, 12] , stabilizing the gut mucosal barrier [13] and modulation of innate immune responses and adaptive [14, 15] .

There are few studies on the probiotic activity of *Lactococcus* which are commonly used in starter cultures for the production of fermented milk products. It has generally been assumed that the *Lactococcus* will not survive passage through the gastrointestinal tract, but studies have reported that some *Lactococcus* strains can survive in the human or animal gastrointestinal tract [16] .

In a recent study, it was demonstrated that *Lactococcus lactis (L. lactis),* NCDO 2118 wild type has a anti-inflammatory activity *in vitro,* in cultured intestinal epithelial cells, as well as *in vivo* in a model of colitis induced by dextran sulfate sodium [17] . Furthermore, Alvarenga et al. [2] found that pretreatment with *L. lactis* NCDO-2118 wild type and secreting Hsp65 in animals who consumed ethanol, reversed classic signs of allergic reaction of type I and impacted differentially on the number of antigen presenting cells activated, such as macrophages, dendritic cells and B lymphocytes in the lymph node mesenteric and spleen.

This study aims to evaluate the effect of *Lactococcus lactis* treatment in allergic inflammation after acute administration of ethanol.

Materials and methods

Animals

C57BL/6 wild strain female mice (8–10 weeks old) were maintained in cages with water and feed (Labina, Purina®) *ad libitum*, at 25 ± 2°C for a photoperiod of 12 hours. All procedures were performed according to the rules set forth in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, MD, 1996). The procedures were approved by the Ethics Committee for Experimentation and Animal Welfare (CEEBEA) under the advice No. 043/13.

Lactococcus lactis

L. lactis subsp. *lactis* wild type strain NCDO-2118 was cultured in M17 broth (Difco, Becton Dickinson) supplemented with glucose (0.5%, m/v), at 30°C without stirring for 18 hours. The M17 broth is a culture medium to isolate lactic streptococci. The suspension of *L. lactis* was calibrated to 10^9 viable bacteria mL^{-1}, equivalent to 0.2 OD at 600 nm wavelength, as measured by a spectrophotometer (Ultrospec 1100 pro; Amersham Biosciences, England). Bacterial suspension (5 mL) was released into bottles for *ad libitum* consumption by the animals, according to the following experimental protocol.

Experimental design

For four consecutive days, intragastric administration of 0.2 mL 50% (v/v) ethanol per animal (ethanol group) was performed. NaCl solution (0.9%, w/v) was used as a treatment control. After 24 hours of administration, the animals were given *L. lactis*, M17 broth or water in bottles for two consecutive days. Shortly after the second day of treatment *ad libitum*, the animals were sacrificed and dissected their stomach, small intestine and liver for histological and immunological analysis and blood samples for immune analysis.

Figure 1A illustrates the sequence of the procedures adopted. The test involved four treatment groups: G1 – animals subjected to saline, treated with M17 broth (Saline and M17); G2 – animals subjected to saline, treated with *L. lactis* (Saline and *L. lactis*); G3 – animals subjected to ethanol, treated with water (Ethanol and water); and G4 – animals subjected to ethanol, treated with *L. lactis* (Ethanol and *L. lactis*). Six animals were randomly chosen from each group for experiments.

Biometric analysis

During the experimental protocol (7 days) the body weight of the animals was assessed, and after euthanasia, also measured the weight (ratio gastric weight / body weight) and stomach size (length of the cardiac ostium to the pyloric ostium).

Histological analysis

Stomach, small intestine and liver were removed, rinsed with 0.9% physiological saline and fixed in 10% formalin in PBS (Phosphate-buffered saline) for 48 hours. After adding the tissue in paraffin, tissue sections of 4μm was obtained and stained with hematoxylin-eosin for mucosa integrity of view, the presence of inflammatory infiltrate and edema in the hepatic parenchyma and in the gastric and intestinal submucosal (proximal jejunum) and toluidine blue for evaluation of mast cells in the gastric submucosa. In hematoxylin-eosin blades three fields/animal images were analyzed under light microscopy (100X). In Toluidine Blue blades, the mast cell count was performed in each gastric extent under light microscopy (400X magnification), and the result was expressed as total number of mast cells. The images were captured from a micro camera Moticam 2500.

Serum total IgE analysis

The collected blood was centrifuged at 600 ×*g* for 10 minutes and the serum obtained was frozen for total immunoglobulin (Ig) E analysis, *a posteriori.* The levels of total IgE antibodies was performed by ELISA (Enzyme Linked Immunosorbent Assay), as described below.

Briefly, 96 well plates (Nunc, Roskilde, Denmark) were coated with 50 μL/well of anti-IgE mouse mouse antibody, diluted 1:200 (rat anti-mouse IgE, Southern Biotechnology, Birmingham, AL) at pH carbonate buffer 9.6. Subsequently, plates were washed twice with 300 μL saline-Tween (0.15M saline containing 0.05% Tween 20) and blocked with 200 μL PBS solution containing 0.25% casein (Sigma Chemical Co., St. Louis, MO, USA) for 1 hour at temperature environment. After washing the plates twice with 300 μL saline-Tween, were added 50 μL of serum, and incubated for 2 hours at room temperature protected from light. After five washings with saline-Tween, were added 100 μL of mouse anti-IgE mouse antibody conjugated to Biotin (rat anti-mouse IgE-BIOT Southern Biotechnology, Birmingham, AL) diluted 1:250 in PBS-casein, and samples incubated for 1 hour at room

temperature. Subsequently, plates were washed five times and incubated for 1 hour with 100 µL of Streptavidin-Peroxidase (Southern Biotechnology, Birmingham, AL) diluted in PBS-casein at a concentration of 1:10,000 for 1 hour at room temperature. Then the plates were washed and incubated, protected from light with 100 µL of citrate buffer (pH 5.0) containing hydrogen peroxide ($H_2O_{2)}$ and 30% orthophenylene diamine (OPD) (1 mg/mL). Thereafter, the reaction was stopped by adding 20 µL of sulfuric acid (H_2SO_4) 2N. The optical density was obtained in ELISA reader (Reader TP, thermoplate, China) with 492 nm filter. The results are expressed as absorbance (OD).

Secretory IgA analysis

The organs, stomach and small intestine were removed and washed with 5 mL and 10 mL of cold physiological saline 0.9%, respectively, for collection of the gastrointestinal lavage in polystyrene tubes. Then, the collected material was vortexed, centrifuged at 450 ×*g* for 20 minutes at 4ºC and the supernatant collected for dosing secretory IgA (sIgA) by ELISA, described below.

Briefly, 96 well plates (Nunc, Roskilde, Denmark) were coated with 100 µL of anti-IgA mouse goat antibody (Goat anti-mouse IgA UNLB; Southern Biotechnology, Birmingham, AL) diluted 1:10,000 in pH carbonate buffer 9.6. After the plates were washed two times with saline-Tween and blocked with 200 µL PBS solution containing 0.25% casein (Sigma Chemical Co., St. Louis, MO, USA) and incubated for 1 hour at room temperature. After washing twice with saline-Tween, 100 µL of washed stomach and intestinal (diluted 1:10) and monoclonal antibodies standards (0.1 µg/mL) (UNLB mouse IgA, Southern Biotechnology, Birmingham, AL) were added to the wells and incubated for 1 hour at room temperature. Subsequently, plates were washed 5 times with saline-Tween and incubated with 100 µL of a solution of anti-IgA mouse goat antibody conjugated to biotin (anti-mouse goat IgA-BIOT; Southern Biotechnology, Birmingham, AL), diluted in PBS-casein 0.25% (1:10,000) for 1 hour at

37°C. After 5 washings, plates were incubated with 100 μL of a solution containing Streptavidin-Peroxidase (Southern Biotechnology, Birmingham, AL), diluted in PBS-casein 0.25% at the concentration of 1:10,000 for 1 hour at room temperature. The plates were washed and incubated, protected from light with 100 μL of citrate buffer (pH 5.0) containing H_2O_2 30% and OPD (1 mg/mL). The reaction was stopped by adding 20 μL of H_2SO_4 2N and read was performed in automatic reader (Reader TP, thermoplate, China) using the filter of 492 nm. The results were expressed in μg/mL.

Cytokine analysis *in situ*

After washing the stomach and small intestine, as described above, the tissues were stored at -80°C until processing. Stomach, duodenum and proximal jejunum were crushed with a mortar and pestle with extraction buffer 1 mL for each 100 mg tissue (buffer: NaCl - 2,34 g%, EDTA - 37,2 mg%, benzotonio chloride - 4,48 mg%, fluoride phenylmethylsulfonyl - 1.7 mg%, bovine serum albumin – 500 mg% in Tween 20 – 5 μL, aprotinin – 10 UIC/mL - Sigma, St Louis, MO, USA). The samples were centrifuged at 1120 ×*g* for 10 minutes at 4°C and the supernatant collected for determination of cytokines by ELISA. Were performed measurements of IL-2, IL-4, IL-10 and interferon gamma (IFN-γ) in the stomach, and dosages of IL-4 and IL-10 in the duodenum and proximal jejunum.

Briefly, 96 well plates (Nunc, Roskilde, Denmark) were coated with 50 μL of anti-IL-2 monoclonal antibodies (2 μL/mL), anti-IL-4 (8 μL/mL) and anti-IL-10 (8 μL/mL) mice (BD Pharmingen, San Diego, CA, USA) diluted in phosphate buffer pH 6.0 (NaH_2PO_4 - 0.1M, $NaHPO_4$) and anti-IFN-γ (2 μL/mL) diluted in carbonate buffer pH 8.3 (Na_2CO_3, $NaHCO_3$ - 0.1 M). Subsequently, plates were washed twice with 300 μL of Saline-Tween and blocked for 1 hour with 200 μL of PBS-casein 0.25% at room temperature. After two washes with 300 μL in Saline-Tween, 50μL of supernatant samples from tissue extracts, and standards for IL-2 (20 ng/mL) and IFN-γ (20 ng/mL) (Pharmingen, San Diego, CA, USA) were added to the

appropriate wells and incubated at 4°C overnight. The next day, after 6 washes with 300 μL saline-Tween, were added 50 μL of monoclonal antibodies conjugated with biotin diluted in PBS-casein 0.25% specific for IL-2 (3 μL/mL), IL-4 (6 μL/mL), IL-10 (3 μL/mL) and IFN-γ (3 μL/mL) of mice (Pharmingen, Becton Dickinson, Mountain View, CA) and incubated for 1 hour at room temperature. The plates were washed 6 times with 300 μL in Saline-Tween and incubated with 50 μL of Streptavidin-Peroxidase (Southern Biotechnology, Birmingham, AL) at a concentration of 1:10,000 diluted in PBS-casein 0.25%, for 45 minutes at room temperature. The plates were washed with 300 μL in Saline-Tween and incubated, protected from light with 100 μL of citrate buffer (pH 5.0) containing H_2O_2 30% and OPD (1 mg/mL). The reaction was stopped by adding 20 μL of H_2SO_4 2N and read was performed in automatic reader (Reader TP, thermoplate, China) using the filter of 492 nm. IL-2 levels and IFN-γ were calculated from a standard curve and results were expressed in ng/mL and IL-4 and IL-10 results are expressed as absorbance (OD).

Statistical analysis

The results were expressed as the mean ± standard error of 6 animals in each experimental group. We used the Kolmogorov-Smirnov test and normal distribution was considered normal if $p > 0.05$. Comparative analysis between groups was performed by ANOVA one way followed by Tukey's post-test and the significance level was 5% ($p < 0.05$). The software GraphPad Prism version 6.0 (GraphPad Software Inc., CA, USA) was used for statistical and drawing the graphs analysis.

Results

Effect of *L. lactis* in biometric and morphological parameters of mice that received prior intake of EtOH

L. lactis treatment not reversed the weight loss observed in animals upon ingestion of EtOH. Interestingly, *L. lactis* treatment was able to reverse the baseline, the increase in gastric size and weight observed in animals previously treated with EtOH as shown in figure 1B.

Macroscopic signs of inflammation were observed in stomach tissues in any experimental groups (data not shown). Furthermore, after administration of EtOH, were observed in gastric and intestinal submucosa (proximal jejunum), microscopic inflammatory changes such as bleeding, increased cellularity and villous atrophy (Figure 1B). There morphometric parameters were evaluated for a quantitative analysis of the inflammatory process, however, it is observed visually that the EtOH caused structural changes in the gastric submucosa.

To investigate the effects of acute EtOH ingestion in the hepatic parenchyma, we observed the presence of extensive areas of hydropic degeneration, represented by the presence of cytoplasmic vacuoles, maintaining the integrity of the core. However, *L. lactis* treatment was able to reduce the degree of degeneration, represented by a lower presence in areas vacuolated hepatocytes (Figure 1B).

Effect of *L. lactis* in immunity of the gastric and intestinal mucosa of mice that received prior ingestion of EtOH

L. lactis treatment reversed the increase in production of sIgA in the gastric mucosa of animals which received EtOH priori. The same effect was not observed the levels of sIgA in the intestinal mucosa of the animals (Figure 2A).

Effect of *L. lactis* in biomarkers associated with allergic responses in mice previously treated with EtOH

It is observed an increase in serum total IgE levels in animals previously ingested EtOH, and a reversal values similar to the control group (saline) after *L. lactis* treatment. As the number of mast cells in the gastric submucosa, it is observed that the administration of alcohol and *L. lactis* treatment did not affect the cell counts (Figure 2B).

The analysis of gastric tissue demonstrates that EtOH administration increased the production of IL-4 and IL-10, but did not affect the production of IL-2 and IFN-γ. *L. lactis* treatment reduced levels of IL-4 and IL-10 did not affect the IL-2 levels, and significantly increased IFN-γ levels (Figure 2B).

The analysis data of the tissue of the small intestine (duodenum and proximal jejunum) demonstrates that *L. lactis* treatment reversed the enhancement of IL-4 EtOH triggered by basal levels and reduced levels of IL-10 (Figure 2B).

Figure 3 summarizes the changes observed after administration of ethanol and *L. lactis* treatment.

Discussion

Few studies have shown the implications of alcohol on immunomodulation, and our group showed loss of susceptibility to the induction of oral tolerance induced by ingestion of ethanol [3] . This mechanism needs to be better understood, but in this same study our group demonstrated that alcohol induces an inflammation of the allergic type in animals and this can compromise the immune homeostasis and phenomena resulting from this balance, as oral tolerance [3] . In this sense, the search for alternative strategies to reduce or mitigate such effects becomes extremely important.

This study was based on a possible immunomodulatory impact achieved by eating a *posteriori* of *Lactococcus lactis,* as a therapeutic strategy of the gastrointestinal mucosa of the animals would receive alcohol.

The results showed that acute ethanol ingestion caused the loss of body weight, increase in weight and size gastric, hepatic degeneration, however caused no damage in the gastric and intestinal mucosa. Previous studies using this same model also observed this alteration in stomach weight and size [2, 3] , and liver [2] after the ethanol consumption. However, after

administration of *L. lactis*, there was a reversal of gastric and liver disorders caused by alcohol. A study of our research group, showed that previous intake of *L. lactis* did not prevent gastromegalia induced acute alcohol intake, but reduced the degree of liver degeneration [2] .

Acute exposure to alcohol in the small intestine mucosa inhibits the active transport of many nutrients across the epithelial layer [18] . The alcohol can lead to a change in intermediary metabolism, and the relationship between alcohol consumption and body weight is a paradox. Small amounts of ethanol appear to have no effect on body weight. The ingestion of moderate amounts can lead to an increase in body weight through a suppressive effect of lipid oxidation. Excess intake of alcohol in leads to a decrease in body weight, probably by enhancing lipid oxidation and energy expenditure [19] . Therefore, it is suggested that excessive intake of alcohol reduces body weight in the inhibition of nutrient absorption, and *L. lactis* treatment for 2 days was not sufficient to improve this absorption and cause the body weight to return to normal.

It was shown that alcoholic solutions above 15% can inhibit gastric motility, delaying emptying of the stomach. With increasing time the gastric transit, bacterial degradation of food starts generating gas and bloating [20] . Thus, the increased weight and gastric size can be explained by accumulation of gases present, checked at necropsy, which was reversed after administration of *L. lactis.*

Even after alcohol consumption and *L. lactis* treatment, this organism survives the gastrointestinal mucosa (data not shown), and operates as a probiotic, it may increase the absorption of minerals and vitamins production.

Several studies show that the intake of ethanol, particularly at high doses and chronic form, triggers alcoholic liver disease. In this study, the presence of cytoplasmic vacuoles in liver of animals treated with EtOH, however was not detected elevation of alanine aminotransferase (ALT) levels (data not shown). ALT is an enzyme whose serum levels are elevated in any

condition that causes acute hepatocellular impairment, and acute or chronic alcohol intake can lead to liver damage with increased serum ALT [21, 22] , but this increase especially in acute consumption depends on the dose and the protocol used. Though only found hepatic degeneration, *L. lactis* treatment reduced this change, demonstrating a therapeutic effect with systemic repercussions.

Studies have shown that *Lactobacillus acidophilus* and *Bifidobacterium longum* ingestion is capable of reducing gastric and especially liver damage caused by acute ingestion (5 days) alcohol. This effect was related to the ability of these microorganisms to reduce the concentration of alcohol in blood by increasing the activity desidrogrenase alcohol, accelerating the metabolism of ethanol in the stomach and the liver [23] . One study demonstrated the beneficial effect of lactic acid bacteria in the liver injury induced by alcohol is associated with maintenance of the intestinal barrier and prevention of endotoxemia [24] . Thus, it can be suggested that the administration of *L. lactis* is able to attenuate liver damage induced by alcohol.

As the gastric and intestinal mucosa, several studies have shown that after ethanol ingestion gastric mucosa of animals showed marked injury. In these studies, it is observed that animals were sacrificed between 30 minutes to 3 hours after the administration of alcohol, and thus suggests that the difference in time between ingestion of EtOH and euthanasia of animals can be a major factor that allows for better visualization of the gastric lesions [25, 26] . In fact, one study found blood clots and linear bleeding on the surface of the gastric mucosa 15 minutes of absolute alcohol administration in rats. The severity of the lesions, showed elevated after 1 hour and stood for up to 6 hours. However 24 hours after alcohol consumption, gastric mucosa was almost completely normal [27] . In our model, the animals were sacrificed 72 hours after the last administration of EtOH, which explains the absence of lesions that given time, and does not exclude the possibility of any damage to the mucosa in the days before euthanasia. The

absence of lesions can be explained due to the high power of the reconstructive gastric mucosa, for many years the regenerative capacity of the mucosa has been recognized as an extremely fast and efficient process [28] . There is some evidence that explain the process of healing of mucosal injury, such as migration and/or proliferation of the gastric epithelium participate in the repair process, and the presence of endogenous factors such as prostaglandins, which trigger, mediating, or regulating regeneration and proliferation of the gastric mucosa in response to acute damage [29] .

Besides affecting the morphology and function of various gastrointestinal components, alcohol can modify a wide variety of immune responses, such as, for example, the production of secretory IgA, an immunoglobulin that protects the gastrointestinal mucosa [30] , preventing infections pathogens and reducing aberrant absorption of allergenic proteins [31] . sIgA is among the most abundant classes of antibodies found in the intestinal lumen [32] , and appear to exert their anti-inflammatory effects, reducing bacterial proinflammatory pathways and limiting the release of cytokines induced by LPS (e.g., IL-1 and TNF). Several studies have shown that the sIgA level is increased in alcoholics which may be a compensatory mechanism of protection, to limit the damage induced by alcohol [33] .

In this study, analysis of the gastric and intestinal mucus has shown that the consumption of ethanol is capable of increasing IgA secretion, as noted in our research group [2] . A study in healthy humans showed that the administration of one dose with a large amount of ethanol caused an increase twice sIgA in saliva compared to samples taken before ethanol consumption [34] . After administration of *L. lactis,* we observed only a reduction of gastric IgA. Thus, it is proposed that *L. lactis* return to homeostasis of the immune system. However, the anticipated intake of *L. lactis* potentiated the effect of alcohol produces more sIgA [2] . In this case, the increase of sIgA can be protecting the lining against the ingress of enteric bacteria, through the epithelium and maintaining the integrity of epithelial junctions.

Experimentally it has been shown by our research group intake of alcohol enables the development of an allergic inflammatory profile, represented by the increase of total serum IgE and IL-4 on gastric and spleen cell cultures [3] . Confirming this study, our results also revealed that alcohol consumption causes increased serum IgE levels and IL-4 gastric, and increases intestinal IL-4 and IL-10 gastric and intestinal.

Mast cells play a fundamental role in increasing vascular permeability, as well as up regulation of adhesion molecules needed for leukocyte recruitment [35] . Being centras in allergic processes cells, it was expected to find an increase in the number of mast cells in the submucosa, which could not be observed in our study. As the animals were sacrificed 72 hours after the last administration of EtOH, this may explain the lack of increase in the number of mast cells of the large local cellular immune regulation, but does not exclude the possibility of the occurrence of changes days prior to euthanasia.

Some studies have linked alcohol consumption to increased serum IgE in which show that many non - atopic alcoholics show an increase in the concentration of serum total IgE when compared to healthy controls [36-38] . However, this increase tends to reduce after abstinence [37] .

One study showed that administration of *L. lactis* wild or genetically modified to secrete IL-10, in an experimental model of allergic anaphylaxis induced by β-lactoglobulin, was able to reduce serum levels of IgE and anti-β-lactoglobulin IgG1 [39] . Some studies show that prior consumption of probiotics, including *L. lactis,* in experimental models of allergies, were able to drastically reduce IL-4 levels in cell cultures of spleen and mesenteric lymph nodes, possibly by regulating the balance between Th1 and Th2 cells [40, 41] .

The alcohol induced changes in the balance of cytokines polarization to production of Th2 cytokines which are correlated with elevated IgE levels in alcoholics [42] and in animal models [43] . It has been reported that alcoholics admitted to a hospital with alcohol

withdrawal syndrome had increased levels of some types of Th2 cytokines compared with healthy controls [38] .

Cytokines produced by Th1 and Th2 control the synthesis of IgE. Th2 cytokines, especially IL-4 and IL-13 are necessary for isotype switching to IgE in B - lymphocytes, in turn, Th1 cytokines, especially IFN-γ, inhibit IgE synthesis [44] .

As IL-4 and IgE are potent inflammatory mediators in allergic reactions, our results suggest that an allergic component can be connected to ethanol consumption. Thus, it was observed that acute alcohol ingestion induces a Th2 inflammatory response.

IL-10 is an important cytokine involved in regulation mechanisms in the gut. Most studies suggest that the predominance of a Th2 profile in the intestinal microenvironment is critical for the establishment of local immunoregulatory events and inflammatory bowel disease developed in IL-10 deficient mice associated with the breakdown of oral tolerance to enteric antigens [45] .

Alcohol interfere with mechanisms that include the induction of anti-inflammatory cytokines such as IL-10 and TGFß. The specific effects depend on the duration of exposure to alcohol. Thus, while acute exposure to ethanol increases the production of IL-10 and TGFß in monocytes and macrophages, chronic alcohol exposure, in most cases, is associated with decreased IL-10 production or prevented from increasing levels of IL-10 for counteracting the excessive production of proinflammatory cytokines [1] .

A study showed that monocyte blood samples obtained from healthy volunteers 24 h after an acute alcohol, showed an increase in IL-10 levels. This study reveals a clear interference of alcohol in IL-10 production by blood monocytes [46] as well as observe an IL-10 enhanced secretion of the gastrointestinal compartment. As the stomach and intestines have immunoregulatory mechanisms, the increase in IL-10 can be a reflection of the compensatory mechanisms of IL-4 and IgE increased by exposure to alcohol.

As observed in our study group that *L. lactis* pre-treatment modulates the inflammatory response induced by alcohol, by reducing serum IgE, IL-4 and IL-10 gastric and intestinal and IL-10 intestinal [2] was evaluated whether treatment with this microorganism also lead to immune response modulation. Thus, it observed similar effects, differing only in the fact of *L. lactis* have reduced IL-10 also in the stomach.

Our results suggest that administering a posteriori *L. lactis* have local and systemic immunomodulation contributing to impact, minimizing allergic effects of alcohol, and suggesting probiotic properties of the *L. lactis*.

Moreover, interestingly, *L. lactis* intake induced an increase of IFN-γ in the stomach after consumption of ethanol, which was not observed any change in these levels in animals consumed only alcohol.

IFN-γ is one of the most potent activators of monocytes and in combination with IL-12 form a key inducer in the development of Th1 type immune responses [47] further assist in reducing the levels of Th2 cytokines, including IL-10 [48] .

A study of our research group showed that acute consumption of alcohol can also trigger a mixed inflammatory response types Th1/Th2 [3] , however, in this study, there was a predominance of Th2 response pathway. Thus, it is suggested that as the *L. lactis* reduces Th2 cytokines, it may have been a stimulus for activation of Th1 pathway, producing proinflammatory cytokines, such as IFN-γ that may have helped in reducing IL-10.

This study opens perspectives for the therapeutic use of *Lactococcus lactis* for the treatment of allergic inflammatory processes elicited by acute ingestion of alcohol. Additional studies in humans are needed to better understand this dynamic relationship between the administration of probiotics and regulation of inflammatory changes in the lining of the gastrointestinal tract.

Competing interests

The authors declare that they have no any competing interests.

Authors' contributions

Letícia Antunes Athayde was involved in experimental procedure, collection of tissue and blood samples, acquisition and analysis of data and writing of the manuscript. Sarah Leão Fiorini de Aguiar, Mariana Camila Gonçalves Miranda and Ronize Viviane Jorge Brito was involved in experimental procedure and collection of tissue and blood samples. Dr. Ana Maria Caetano de Faria contributed to the study design and interpretation of data. Dr. Mariléia Chaves Andrade and Dr. Sergio Avelino Mota Nobre was involved in the design and coordination of the project, the acquisition, analysis and interpretation of the data.

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Figures and figure legends

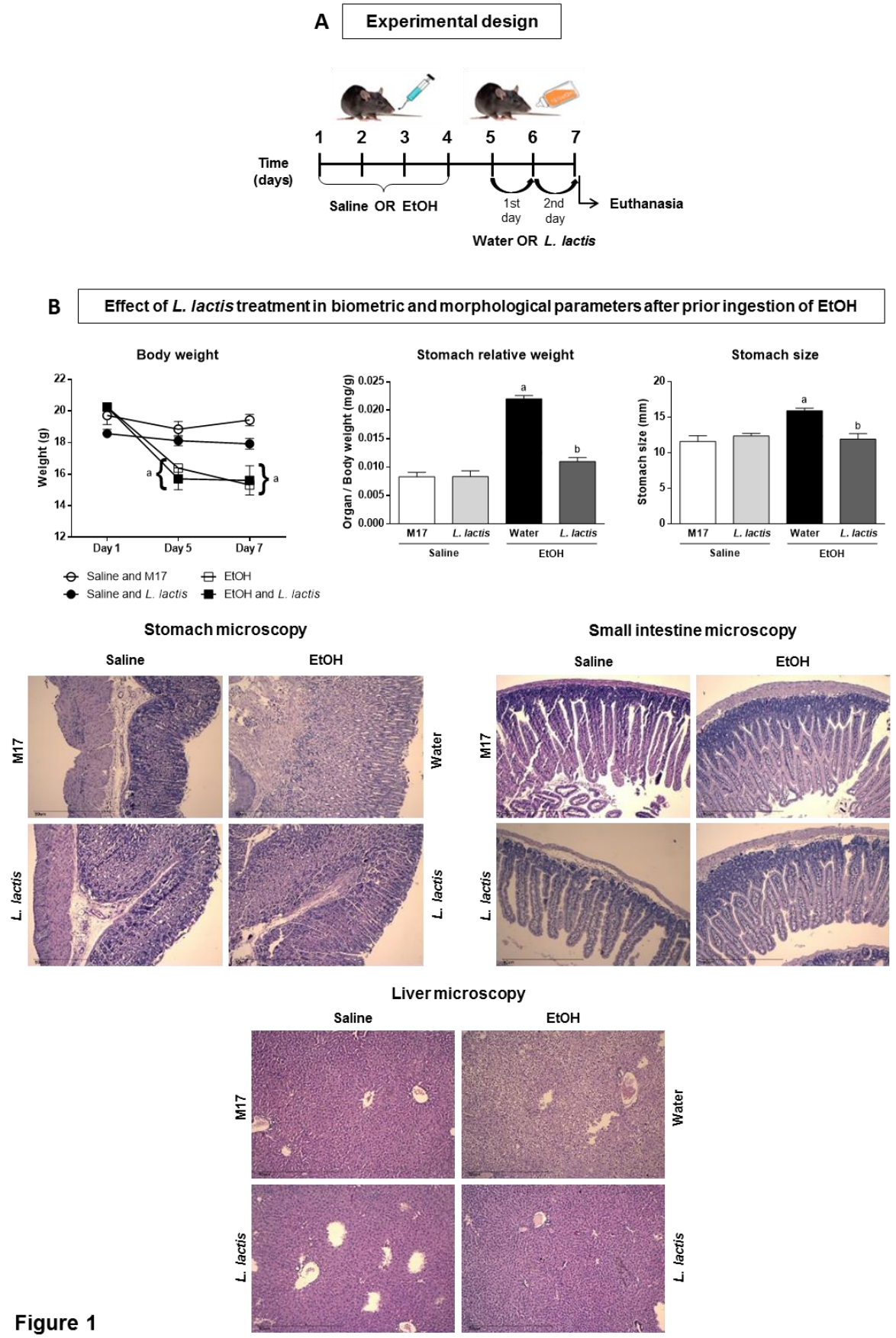


Figure 1

Figure 1. Experimental design for evaluating the effect of *L. lactis* treatment in biometric and morphological parameters of mice that received prior ingestion of EtOH. (A) Experimental design: for four consecutive days, C57BL/6 female mice (n = 6) were given 50% (v/v) ethanol (EtOH) or saline solution by gavage. After 24 hours of last administration, the animals were administered *Lactococcus lactis*, M17 broth or water orally *ad libitum* for two consecutive days. Shortly after the second day of treatment, the animals were sacrificed, and their blood, stomach and small intestine were collected for immunological and histological analysis. (B) The body weight, size and weight on stomach along with histology of the stomach, small intestine (proximal jejunum) and liver (microscope) were evaluated. Results are expressed as mean ± standard error of the mean. Significant differences at $p < 0.05$ are indicated by "a" when compared to saline and M17 and saline and *L. lactis* groups, and "b" when compared to the EtOH and water group.

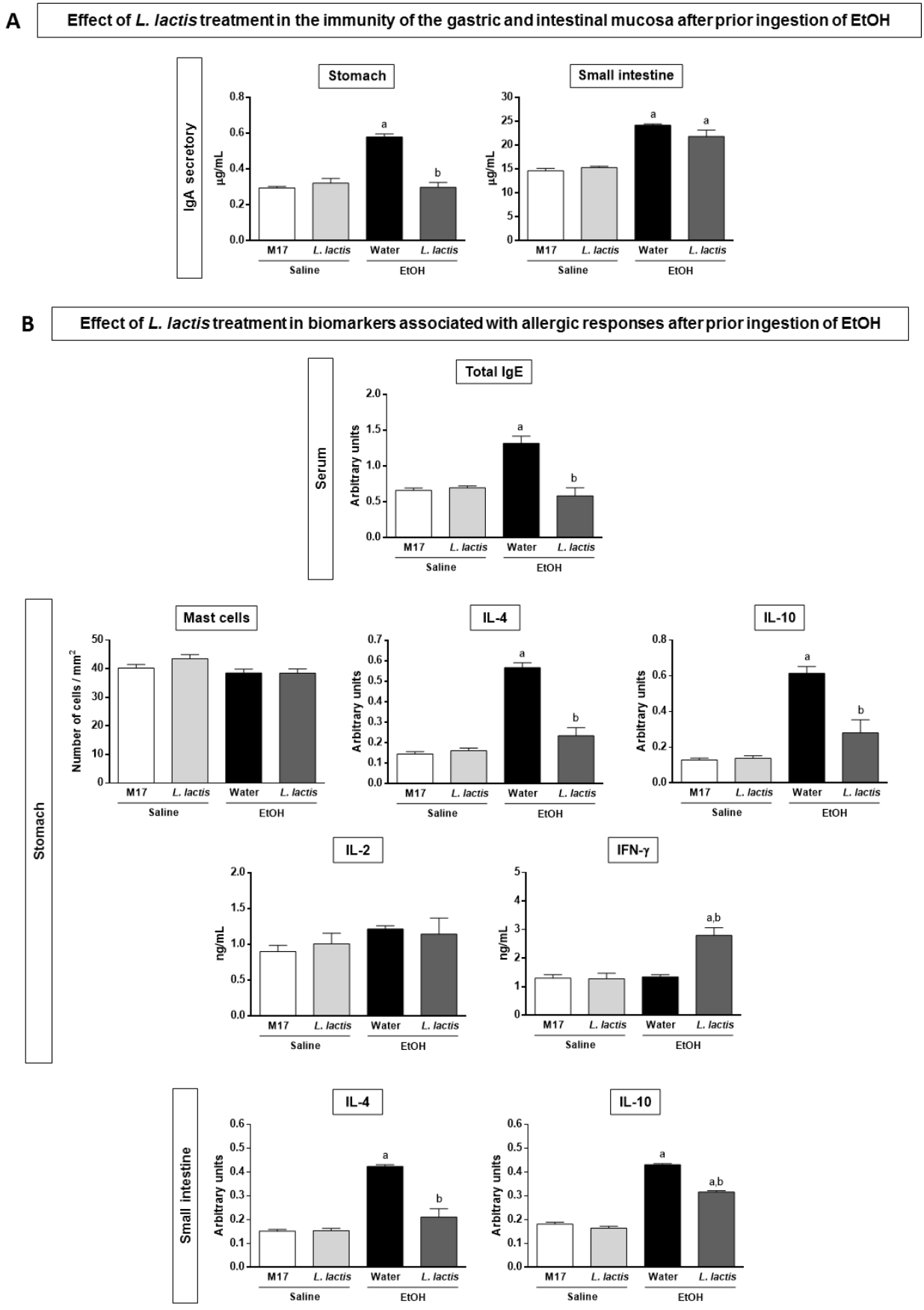

A
Effect of L. lactis treatment in the immunity of the gastric and intestinal mucosa after prior ingestion of EtOH
IgA secretory
Stomach
Small intestine
µg/mL
M17
L. lactis
Water
L. lactis
Saline
EtOH
B
Effect of L. lactis treatment in biomarkers associated with allergic responses after prior ingestion of EtOH
Serum
Total IgE
Arbitrary units
Stomach
Mast cells
Number of cells / mm2
IL-4
IL-10
IL-2
IFN-γ
ng/mL
Small intestine

Figure 2

Figure 2. Effect of *L. lactis* in immunity of the gastric and intestinal mucosa and biomarkers related to allergic responses in mice that received prior intake of EtOH. According to the experimental design, right after the end of the second day of *L. lactis* treatment, were collected washed gastrointestinal IgA analysis secretion by ELISA (A), serum samples for quantification of total IgE by ELISA, stomach samples mast cell counting after staining with Toluidine Blue, and IL-4 analysis IL-10, IL-2 and IFN-γ by ELISA, and samples of the small intestine (duodenum and proximal jejunum) for analysis of IL-4 and IL-10 by ELISA (B). Results are expressed as mean ± standard error of the mean. Significant differences at $p < 0.05$ are indicated by "a" when compared to saline and M17 and saline and *L. lactis* groups, and "b" when compared to the EtOH and water group.

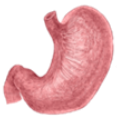
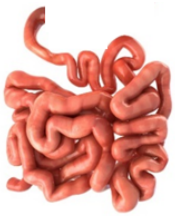
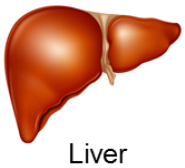
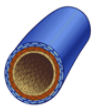
	EtOH - water	EtOH - *L. lactis*
 Stomach	↑↑ weight ↑↑ size ↑↑ sIgA ↑↑ IL-4 ↑↑ IL-10	↓↓ weight ↓↓ size ↓↓ sIgA ↓↓ IL-4 ↓↓ IL-10 ↑↑ IFN-γ
 Small intestine	↑↑ sIgA ↑↑ IL-4 ↑↑ IL-10	↑↑ sIgA ↓↓ IL-4 ↓ IL-10
 Liver	Degeneration hydropic	↓ Degeneration hydropic
 Serum	↑↑ Total IgE	↓↓ Total IgE

Figure 3

Figure 3. Summary of alterations observed after administration of ethanol and *L. lactis* treatment. Data from EtOH group - water that are presented were obtained when compared with the control group saline. In turn, data from the EtOH - *L. lactis* group were observed when compared to group EtOH - water.

Effect of *Lactococcus lactis* treatment on *Enterobacteriaceae* and lactic acid bacteria populations in the gastrointestinal tract after ethanol ingestion

Letícia Antunes Athayde,[a] Ronize Viviane Jorge Brito,[a] Mariléia Chaves Andrade,[a] and Sergio Avelino Mota Nobre,[a]*

[a] Universidade Estadual de Montes Claros, Montes Claros, MG, Brazil.

Address correspondence to Sergio Avelino Mota Nobre, sergio.nobre@unimontes.br

*Laboratório de Epidemiologia e Biocontrole de Microrganismos, Universidade Estadual de Montes Claros, CP 126, Montes Claros-MG, Brazil.

Tel: +55 38 3229-8189

Abstract

Alcohol is a major component of diet in Western societies and can potentially influence the intestinal microbiota. Several studies have shown that excessive alcohol consumption alters the composition of intestinal microbiota in rodents and humans, disrupting the microbiota homeostasis. The aim of this study was to evaluate the effect of *Lactococcus lactis* treatment on the populations of gram-negative *Enterobacteriaceae* bacteria and lactic acid producing gram-positive bacteria in the gastrointestinal tract (GIT), after acute administration of ethanol. C57BL/6 females mice received ethanol or saline solution by gavage for four consecutive days, and 24 hours after the last administration, the animals were given *L. lactis* or M17 broth orally *ad libitum* for two consecutive days. After this, the animals were sacrificed and dissected. Their stomach, small intestine, and large intestine (colon) sections were washed with sterile saline and microbiological sampling was conducted. Ingestion of ethanol by animals strongly detached *Enterobacteriaceae* from the stomach mucosa and small intestine and suppressed presumptive lactic acid bacteria and presumptive *L. lactis* populations in the GIT. *L. lactis* treatment encouraged the diversification of *Enterobacteriaceae* population, particularly the commensal species, in the GIT. Our findings show the existence of direct and indirect effects of ethanol on the gastric and intestinal mucosa, limiting the adherence of the *Enterobacteriaceae* family bacteria. Additionally, *L. lactis*, acting as a probiotic, became more conducive to this environment, supporting colonization and diversification of *Enterobacteriaceae* in the GIT, which were able to survive and colonize the gastric and intestinal mucosa.

Importance

Clinically, the alcohol habitual consumption incurs on negative effects on different organs, including the gastrointestinal tract (GIT). The food manufacturing process has caused

biological exclusions on probiotic bacteria, thereby reducing the natural inclusion of these microorganisms into the human GIT. The alcohol abusive consumption associated with a diet based on foods sterilized by industrialization process can restrict the contact with microorganisms able to maintain the homeostasis of the microbial niches of intestine. The increase of population life expectancy require for more scientific and technological knowledge, as the recognition of biotherapeutic agents and their mechanisms of action. Lactic acid producing bacteria (LAB) are already components of food and pharmaceutical formulations. The assessment of *Enterobacteriaceae* and LAB population parameters of continuous sites of TGI from experimental animals subjected to intake of alcohol and treated with *Lactococcus lactis lactis*, can contributes to the improvement of biotherapeutic use in situations of alcoholic disorders.

Introduction

The digestive microbiota, along with its host, is one of the most complex ecosystems, but less known and controlled. There are two microbial groups in this ecosystem: the indigenous and allochthonous microbiota. The indigenous microbiota is made up of resident microorganisms at stable population levels in an anatomical site or at specific developmental stages of the individual's life. The allochthonous microbiota is found sporadically and transitorily at any anatomical site, where microorganisms are acquired through ingestion of foods and beverages, through the skin, or upper respiratory membrane (1).

The intestinal microbiota has three major roles in host health: resistance to colonization (inhibits proliferation of non-native and opportunistic native pathogens), immunomodulation (allows a more rapid and proper immune response during an infectious disease), and nutritional contribution (provides vitamins and energy substrates) (2, 3). Moreover, the presence of intestinal microbiota plays an important role in maintaining the mucosal structure (4).

Commensal microbial communities in the intestine are highly diverse at species level, but have low diversity at phylum level. In all vertebrates, intestinal commensal microbiota is dominated by two phyla: *Bacteroidetes* (gram-negative) and *Firmicutes* (gram-positive), comprising about 90% of the phyla in the intestine. The other 10% of the total population belongs predominantly to the phyla *Proteobacteria* and *Actinobacteria* (5).

The bacteria belonging to the phylum *Proteobacteria* are normally detectable in gastrointestinal samples, and this group of gram-negative bacteria is particularly diverse, although not very abundant, contributing to about 1% of the total microbiota (6). There are five different classes of *Proteobacteria*, namely *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, and *Epsilonproteobacteria*, among which *Enterobacteriaceae,* of *Gammaproteobacteria*, is the most abundant and predominant family. Most members of *Enterobacteriaceae* are associated with diarrhea (7), although the representative bacteria of this family are not associated with any symptoms and are pioneers in the gastrointestinal tract (GIT) of infants (8). *Escherichia coli*, the most frequent representative of this family, are often the most abundant facultative anaerobic bacteria in gastrointestinal samples. Different strains of *E. coli* may exhibit different properties, ranging from probiotics (9) to pathogens causing diarrhea and infections (10). Most of the other bacteria belonging to the *Enterobacteriaceae* family are rarely isolated from gastrointestinal samples (6).

The dysbiosis phenomenon, caused by disturbance in homeostasis of the intestinal microbiota, has been associated with irritable bowel syndrome (11, 12), celiac disease (13), and food allergies (14). Although it is unclear whether dysbiosis is the cause or result, the factors that contribute to the development and progression of many of these diseases are known to influence the microbiota of the GIT (15).

Dysbiosis can be caused by environmental factors commonly found in Western societies, including diet (16), circadian rhythm disturbance (17), and alcohol consumption (18, 19). It is

well established that diet influences the composition and diversity of the intestinal microbiota (16).

Alcohol is one of the main components of diet in Western societies and can potentially alter the intestinal microbiota. Several studies in rodents and humans have shown that excessive consumption of alcohol alters the composition of the intestinal microbiota, causing disruption of microbiota homeostasis (18–21).

Most studies indicate an association between intestinal bacterial growth induced by alcohol, and dysbiosis, and the development of alcoholic liver diseases and cirrhosis (15). Alcohol breaks down the intestinal barrier (22) by increasing oxidative stress in the intestine, which in turn disturbs the tight junction and promotes intestinal hyperpermeability (23). Increased intestinal permeability allows the translocation of microbial proinflammatory products, including endotoxins, such as lipopolysaccharide and peptidoglycan, and pathogens from the intestinal lumen into the liver via the portal vein (24). Exposure to these bacterial products, which cause inflammation of the liver, in association with the direct effects of alcohol may cause alcoholic liver disease (25).

Restoring homeostatic composition of the intestinal microbiota can be a corrective measure for the changes caused by excessive alcohol intake. Probiotics, prebiotics, and synbiotics are ways to intervene in the intestinal microbial ecosystem (26), and scientific researches are enabling complete reversal of alcohol-induced intestinal dysbiosis and its consequences, using probiotics.

Studies in rodents have shown that probiotics prevent alcohol-induced dysbiosis. A study in C57BL/6 mice given alcohol (5%, v/v) orally for 6 weeks showed that oral administration of *Lactobacillus rhamnosus* GG (10^9 CFU mL^{-1}) for 6 to 8 weeks prevented ethyl dysbiosis (20).

Lactobacilli and bifidobacteria are most commonly used as probiotics, although other microorganisms, such as *Lactococcus lactis*, are also potent probiotics, being able to restore microbiota homeostasis and enhance innate defenses, such as intestinal immunoglobulin A secretion. Scientists have used these immunomodulatory *L. lactis* to protect the gastrointestinal mucosa of animals receiving alcohol (27); however, the effects of these bacteria on the intestinal microbiota of animals are still obscure.

This study aimed to evaluate the effects of *L. lactis* active treatment on the structure and abundance of *Enterobacteriaceae* family gram-negative bacteria and gram-positive lactic acid bacteria (LAB) in the GIT of experimental animals subjected to acute ethanol ingestion.

Materials and Methods

As inflammatory response was observed in the GIT after consumption of ethanol (data not shown), we proceeded to investigate the association between ethanol intake and likely intestinal dysbiosis, which changes *Enterobacteriaceae* populations. We also analyzed the effect of *L. lactis* on other likely LAB populations in the GIT.

Experimental animals

C57BL/6 wild strain female mice (8–10 weeks old) were maintained in cages with water and feed (Labina, Purina®, São Lourenço da Mata, Brasil) *ad libitum*, at 25 ± 2°C for a photoperiod of 12 hours. All procedures were performed according to the rules set forth in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, MD, 1996). The procedures were approved by the Ethics Committee for Experimentation and Animal Welfare (CEEBEA) under the advice No. 043/13.

L. lactis

L. lactis subsp. *lactis* wild type strain NCDO-2118 was cultured in M17 broth (Difco, Becton Dickinson) supplemented with glucose (0.5%, m/v), at 30°C without stirring for 18 hours. The suspension of *L. lactis* was calibrated to 10^9 viable bacteria mL^{-1}, equivalent to 0.2 OD at 600 nm wavelength, as measured by a spectrophotometer (Ultrospec 1100 pro; Amersham Biosciences, England). Bacterial suspension (5 mL) was released into bottles for *ad libitum* consumption by the animals, according to the following experimental protocol.

Experimental design

For four consecutive days, intragastric administration of 0.2 mL 50% (v/v) ethanol per animal (ethanol group) was performed. NaCl solution (0.9%, w/v) was used as a treatment control. After 24 hours of administration, the animals were given *L. lactis* or M17 broth in bottles for two consecutive days. Shortly after the second day of treatment *ad libitum*, the animals were sacrificed and dissected their GIT sections were washed with sterile saline. Three sites: the stomach, small intestine, and large intestine (colon), were separated for microbiological analyses.

Figure 1 illustrates the sequence of the procedures adopted. The test involved four treatment groups: G1 – animals subjected to saline, treated with M17 broth (Saline and M17); G2 – animals subjected to saline, treated with *L. lactis* (Saline and *L. lactis*); G3 – animals subjected to ethanol, treated with M17 broth (ethanol and M17); and G4 – animals subjected to ethanol, treated with *L. lactis* (ethanol and *L. lactis*). Three animals were randomly chosen from each group for experiments.

Measuring the populations of *Enterobacteriaceae* and presumptive LAB

Immediately after sacrificing the animals, their stomach, small intestine (duodenum, jejunum, and ileum), and large intestine (colon) were removed and washed with 5, 10, and 5 mL 0.9% physiological saline, respectively. Then, 28.27 mm^2 sections from the stomach tissue,

duodenum, jejunum, ileum, and colon were collected in polystyrene tubes containing 500 µL saline solution. The tubes were centrifuged at 150 ×*g* for 1 minute, for dispersion of the microorganisms. After decimal serial dilutions (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}), 10 µL aliquots of the dilutions were plated on MacConkey Agar (Oxoid, Hampshire, UK) and deMan, Rogosa, and Sharpe agar (MRS agar; Acumedia, Michigan, USA). The tissue washings and the tissues were seeded separately. Samples were taken in triplicates and the plates were incubated at 37°C for 24 hours, the MRS agar being incubated under microaerophilic conditions. The morphological characteristics of the colonies were evaluated using a stereoscopic microscope (SMZ800 Model; Nikon Instruments Inc., Tokyo, Japan), for differentiation of microorganisms. Colony count was carried out using the ImageJ software (Java-based image processing program developed at the National Institutes of Health).

Identification of *Enterobacteriaceae*

Pure cultures of the colonies obtained from the GIT of the animals were collected and preserved at −80°C. For *Enterobacteriaceae*, each isolate was taxonomically identified using the RapID™ ONE System biochemical kit and ERIC® software (Remel, Thermo Scientific, Lenexa, Kansas, USA). Information, such as oxidase enzyme activity and lactose fermentation, were analyzed.

Analysis of presumptive *L. lactis* population

For *Enterobacteriaceae*, pure cultures of colonies obtained from the GIT of the animals were collected and preserved at −80°C. Existing colonies were grown on MRS agar and were subjected to Gram staining. Colonies with morphologies typical of *L. lactis* were recorded as presumptive colonies of *L. lactis.*

Statistical analysis

Colonization intensities were expressed in terms of log CFU mm^{-2} or log CFU mL^{-1}. For each sample record, statistic position, dispersion, and confidence interval (CI: n = 3; $\alpha = 0.05$) were generated. Three animals from each experimental group were used, which were quantitated as three microbiological samples per section of the GIT. Presupposition statistics for parametric analysis were observed and met, using two-way analysis of variance followed by Tukey's post-hoc test. The significance level was set at 5% ($p < 0.05$). GraphPad Prism software version 6.0 (GraphPad Software Inc., CA, USA) was used to make graphs and for statistical analysis. Total bacterial counts were expressed as the colonies from tissues washed with saline and desorbed by centrifugation.

Results

Effect of *L. lactis* on the abundance and diversity of *Enterobacteriaceae* species in the stomach, after ethanol intake

It was observed that biotherapy with *L. lactis* favored the growth of *Enterobacteriaceae* populations in the stomach ($p \leq 0.05$). When considering the effect of ethanol pretreatment on *Enterobacteriaceae* counts, it was observed that ethanol administration followed by M17 broth treatment resulted in significantly low bacterial counts ($p \leq 0.05$). However, post-treatment with viable *L. lactis* did not significantly change the *Enterobacteriaceae* populations in the stomach ($p > 0.05$; Fig. 2A).

The results presented in Figure 2B provide a relationship between biophysical adsorption of the bacterial cells and the mucous in the stomach of animals. It could be inferred that ethanol ingested by animals strongly detached the *Enterobacteriaceae*, and desorption of bacteria and

mucosa via centrifugation led to higher bacterial counts in the washings than in the tissue samples ($p \leq 0.05$).

Some factors related to the gastric mucosa that might influence the microorganisms in it are: (1) the gastric epithelium and mucosa are in continuous movement, stimulating food and debris, and (2) bacteria adsorb on to the mucosal lining via bacterial proteins (adhesins) combined with sugar or lipids, which determine colonization and medium of interaction between the bacteria and host cell surface (28). Ethanol as a solvent might be changing these interactions, and hence, the adsorption of bacteria on to the mucosa.

The *Enterobacteriaceae* species diversity was more significant in the mucosal samples than in the stomach washings, and it was more evident in animals treated with *L. lactis.* There was a selective effect of M17 broth, regardless of pretreatment with saline or ethanol, in favor of fecal coliforms, particularly *E. coli* (Figs. 2C and 2D). In stomach samples, it was observed that pretreatment with ethanol followed by *L. lactis* treatment favored *Enterobacter cloacae*, *Klebsiella pneumoniae*, and *Pseudomonas oryzihabitans* (Fig. 2D), which were also observed in the gastric mucosa (Fig. 2C).

Effect of *L. lactis* on the abundance and diversity of *Enterobacteriaceae* species in the small intestine, after ethanol ingestion

Based on the total colonies recovered from the small intestine samples, it was observed that administration of *L. lactis* stimulated the growth of *Enterobacteriaceae*, which was more evident in animals pretreated with ethanol ($p \leq 0.05$; Fig. 3A), being more marked in the jejunum section (Fig. 3B). Similar to the observations in the stomach, ethanol facilitated detachment of *Enterobacteriaceae* from the mucosa of the small intestine ($p \leq 0.05$; Fig. 3B). In addition, a suppressive effect of these bacteria was observed on post-treatment with M17 broth, just as in the stomach.

As was observed in the stomach, the most prevalent species in mucosal samples and washings of small intestine were several, in animals treated with LAB *L. lactis.* In all mucosal sections, the results indicated a selective effect of M17 broth, regardless of pretreatment with saline or ethanol, favoring fecal coliform bacteria, particularly *E. coli* (Figs. 3C, 3D, and 3E). In the different sections of the small intestine from animals pretreated with ethanol and post-treated with *L. lactis*, *Kluyvera* species was present in the duodenal mucosa (Fig. 3C), *P. oryzihabitans* in the jejunal mucosa (Fig. 3D), and *E. cloacae* in the ileal mucosa (Fig. 3E).

We observed a similar pattern of *Enterobacteriaceae* species diversity in the washings and mucosal samples of small intestine sections from animals post-treated with *L. lactis*; however, the pattern varied between the sections of the small intestine. There was a pattern similarity between the duodenal and ileal mucosa and washings from animals treated with saline and *L. lactis*, unlike the pattern observed in the jejunum from animals treated with ethanol and *L. lactis.* This showed that the presence of *L. lactis* or its metabolites affected the *Enterobacteriaceae* populations in the small intestine (Figs. 3C, 3D, and 3E).

Effect of *L. lactis* on the abundance and diversity of *Enterobacteriaceae* species in the large intestine (colon), after ethanol ingestion

Ethanol ingestion resulted in quantitative different in animals treated with *L. lactis* and opposite phenomenon was observed in animals treated with M17 broth, regardless of prior intake of ethanol ($p \leq 0.05$). Among the animals that ingested ethanol, no significant difference between the scores resulting from treatment with M17 broth and *L. lactis* was observed (Fig. 4A).

The bacterial counts in the washings and colon mucosa samples did not provide a coherent understanding of the peeling effect of ethanol on the *Enterobacteriaceae* populations. One of the factors that might have contributed to this result is the retention of dehydrated stool, which might have reduced the recovery of the microbiota not adhered. However, it was observed that

drunken animals had higher *Enterobacteriaceae* counts when subjected to *L. lactis* treatment than M17 broth treatment. Among animals pretreated with ethanol, treatment with *L. lactis* was not statistically superior to treatment with M17 broth, based on lavage sample scores. Reverse effect was observed for mucosa samples from animals given these treatments (Fig. 4B).

The greatest diversity was observed in animals treated with ethanol and *L. lactis.* There was a combined effect of alcohol consumption and LAB treatment on the diversity of *Enterobacteriaceae* species, being more evident in drunken animals (Figs. 4C and 4D).

Effect of *L. lactis* on the abundance of presumptive LAB in the GIT, after ethanol ingestion

It was noted that ethanol altered the colonization intensity of LAB in the stomach, small intestine, and colon, functioning largely as a repressor of these populations. Under this condition, there was greater variation in viable colony counts. Regardless of the pretreatment, a larger presumptive LAB count was observed in the small intestine, being significantly large in the ileum section, followed by the colon and stomach to a lesser extent (Fig. 5).

In the large intestine, the effects of ethanol on the samples and consequently the LAB population counts were not statistically different between washings and mucosa samples, although greater number of LAB was observed. In animals not drunk, this trend was not evident, showing a more complex relationship with the GIT section (Fig. 5F).

Several studies on the adhesiveness of LAB to the GIT mucous membranes have been conducted and this property is considered an important adaptation attribute of a probiotic. It can be inferred that such a mechanism is isolate-dependent (29).

Presumptive *L. lactis* isolation frequency in the GIT of mice pretreated with ethanol

In animals pretreated with ethanol, a significant decrement in LAB *L. lactis* population was observed in the GIT sections, except in the ileum section of the small intestine (Table 1), suggesting the presence and/or competition between LAB in this fraction of the GIT (Fig. 5D).

Figure 6 summarizes the changes observed in the GIT sections after ethanol administration and *L. lactis* treatment.

Discussion

Studies in rodents and humans have shown that alcohol intake stimulates bacterial overgrowth and intestinal dysbiosis. However, the use of probiotics, especially *Lactobacillus*, as biotherapy is able to reverse this situation, providing an interesting alternative for the treatment of alcohol abuse. Despite the variety of such studies, it is necessary to review the effect of other probiotics, such as *L. lactis*, on the gut microbiota.

In this study, ethanol ingestion by animals caused a more pronounced release of *Enterobacteriaceae* from the stomach mucosa and small intestine and in part suppressed LAB populations throughout the GIT.

Ethanol exerts some direct and indirect effects on gastric physiology. The intake of alcohol reduces gastric motility in a manner not strictly dose-dependent (30), but the motility is primarily influenced by non-alcoholic compounds of the alcohol. Fermented drinks prolong gastric emptying by more than one equivalent of ethanol solution, whereas for distilled spirits, the difference is not significant (30). Only alcoholic beverages produced by fermentation seem to increase gastric acid secretion (31), and this effect is most likely due to non-alcoholic compounds, such as succinic acid and maleic acid (32).

Most of the ingested ethanol is absorbed by passive diffusion through the duodenal and jejunal mucosa, proceeding from the duodenum to the ileum. The intraluminal concentration of ethanol, as a gradient across the epithelial lining, decreases progressively. These observations suggest that the interaction between ethanol and intestinal mucosa is more pronounced in the upper tract of the small intestine, where the intraluminal concentration of ethanol is higher and a significant portion of it is absorbed (33). Additionally, acute ethanol ingestion is associated with erosion of the intestinal villi apex, basal layer separation of the epithelium with the formation and later breaking of bubbles, and subepithelial disruption of the epithelial barrier. These effects are transient, because epithelial regeneration allows for full compensation of the damage within 24–48 hours (34).

Increased intestinal permeability in alcoholics is associated with two important consequences: increased translocation of macromolecules from the lumen to the blood (35) and reduced absorption capacity of the mucosa, associated with the luminal secretion of the intestine being more pronounced (33).

Alcohol interferes with the absorption of nutrients, such as glucose, amino acids, and lipids, and micronutrients, such as folic acid, which is essential for proper maturation and function of intestinal epithelial cells, thereby, creating a vicious cycle. These effects are transient. In fact, alcohol abstinence is associated with complete restoration of the epithelial morphology and function of the intestine (34).

From the results of previous studies, it was concluded that ethanol has a toxic effect on the mucosal epithelium. Based on extensive experimental studies, researchers have proposed an indirect effect of alcohol on the mucosal microcirculation that leads to an enhanced transcapillary fluid filtration and subsequent disruption of the epithelial lining fluid (33). In additional, studies by the same group provided evidence that alcohol induces contraction of the villi, which leads to bubble formation at villi ends, lymphatic obstruction, and, optionally,

exfoliation of the villi tips when the bubble ruptures. These authors also suggested that the initial event in response to alcohol is an increased influx of leukocytes, leading to an increased release of toxic mediators, such as reactive oxygen species and leukotrienes and histamines from mast cells.

Given the above, it has been suggested that the direct and indirect effects of ethanol on the gastric and intestinal mucosa affect the adherence of the microbiota in the mucosa, facilitating the detachment of *Enterobacteriaceae*.

From studies in animals, there have been recent advances in the understanding of the effect of alcohol administration on the amount and composition of the intestinal microbiota. Using an interesting design based on genetic analysis of intestinal microbiota, Yan *et al*. (19) investigated the changes in the intestinal microbial community of mice that were given alcohol for 3 weeks. They observed a relative abundance of *Bacteroidetes* and *Verrucomicrobia* and reduction in the population of *Firmicutes* (*Lactococcus*, *Pediococcus*, *Lactobacillus*, and *Leuconostoc* genera) after alcohol consumption. A study based on the metagenomic analyses of the intestinal microbiota showed a decline in *Bacteroidetes* and *Firmicutes* populations in alcohol-treated mice, with an increase in gram-negative *Proteobacteria* and gram-positive *Actinobacteria* populations (20).

Previous studies (26) and a study by our research group, on acute ethanol intake (data not shown), show that ethanol leads to an increase in *Enterobacteriaceae* populations. However, in this study, we observed that animals administered alcohol and treated with M17 broth, showed decrease in *Enterobacteriaceae* populations, especially in the stomach, and selectively favored *E. coli* throughout the GIT, showing that M17 broth behaves as a suppressor of this group of bacteria.

M17 broth is composed of peptone and meat, as sources of carbon, nitrogen, vitamins, and minerals; yeast extract, as a source of vitamin B, to stimulate the growth of bacteria; β-glycerophosphate disodium salt, for the acid that is produced from lactose fermentation; ascorbic acid, which stimulates the growth of lactic streptococci; and magnesium sulfate, which provides essential ions for growth. Furthermore, at the time of preparation, 10% lactose is added to the medium. The β-glycerophosphate disodium salt, present in greater quantities in the M17 broth, is a phosphate group donor and acts as a protein phosphatase inhibitor. Shankar and Davies (1977) found that β-glycerophosphate disodium in M17 broth suppresses *Lactobacillus bulgaricus* and *Streptococcus thermophilus* isolated selectively from yogurt.

The results of this study showed that treatment with *L. lactis* favored *Enterobacteriaceae* populations and caused greater diversity of this group of bacteria in the stomach, small intestine, and large intestine, showing the presence of *E. cloacae*, *P. oryzihabitans*, *Kluyvera* species, *Enterobacter amnigenus* (normal inhabitants of the GIT), and *K. pneumoniae* (can cause a severe form of pneumonia). Although there was an increase in the population and diversity of *Enterobacteriaceae* in the GIT of animals, it was observed that *L. lactis* induced higher proliferation of these commensal microorganisms.

Several studies have shown that probiotics may induce or inhibit changes in the composition and diversity of microbial species in the intestine. VSL#3, a probiotic mixture of lactobacilli (*Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus acidophilus*, and *Lactobacillus delbrueckii* subspecies *bulgaricus*), bifidobacteria (*Bifidobacterium infantis*, *Bifidobacterium longum*, and *Bifidobacterium breve*), and *Streptococcus salivarius* subspecies *thermophilus*, induced an increase in bacterial populations (mainly lactobacilli and bifidobacteria) and reduced diversity of fungi, as compared to the subjects treated with placebo. This increase in bacterial diversity was not caused by colonization of bacterial strains contained in VSL#3.

However, it is not conclusive whether the anti-inflammatory effects of probiotics are primary or secondary to the induction of changes in the diversity of mucosal microflora.

In a study, a mixture of lactobacilli *(L. rhamnosus* GG, *L. plantarum* CIP102021, *L. casei* CIP107868, and *L. delbrueckii* subspecies *lactis* CIP101028) and bifidobacteria (*Bifidobacterium bifidum* CIP56.7, *B. infantis* CIP64.67, *Bifidobacterium lactis* CIP105256, and *Bifidobacterium adolescentis* CIP64.59) were used to improve colitis induced by dextran sulfate sodium in mice (36). This study showed that the levels of *Bifidobacterium*, *Bacteroides*, and *L. acidophilus* decreased significantly in mice with colitis, compared to controls or group treated with probiotic. Interestingly, although the probiotic mixture used did not contain *Prevotella*, *Bacteroides*, and *Porphyromonas* species, the animals receiving the combination showed normalization of probiotic bacteria levels in these groups. The authors suggest that maintaining levels of bacteria in the colon by probiotics may have induced changes in the luminal metabolism, leading to an anti-inflammatory effect.

In order to study the impact of administration of exogenous *Lactobacillus* strains, commonly used as probiotics, on endogenous microbial populations, Fuentes *et al*. (37) fed *L. casei* and *L. plantarum*, isolated from commercially available dairy products, to mice. The authors reported an increase in the diversity of intestinal lactobacilli (other than *L. casei* and *L. plantarum)* in the feces as well as in the intestinal samples from the mice.

Probiotics may also inhibit the growth of pathogens by production of antimicrobial compounds or reduce their impact through competitive exclusion to occupy binding sites on the surface of the mucosa. For example, *Lactobacillus johnsonii* La1 competes with various pathogens for the same binding sites in the intestine. This competition for binding sites on the mucosal surface can also be the mechanism by which *L. casei* Shirota and *L. rhamnosus* GG displace enterovirulent *E. coli* and *Salmonella enterica* Caco-2 in human intestinal mucus, *in vitro* (38).

Intestinal bacteria are able to produce a variety of vitamins, synthesize all essential and non-essential amino acids, and perform bile biotransformation (39). Furthermore, the microbiota provides vital biochemical pathways for the metabolism of non-digestible carbohydrates, including large polysaccharides, some oligosaccharides that escape digestion, sugars and alcohols not absorbed from the diet, and host-derived mucins (40). This functionality results in energy recovery from the host substrate and a supply of energy and nutrients for host and bacterial growth (41).

Many intestinal bacteria produce antimicrobial compounds and nutrients and compete for binding sites on the gut lining, preventing colonization of pathogens. This action is known as a barrier or competitive exclusion effect. The host cells in the intestinal wall have binding sites that can be used by pathogenic bacteria to enter the epithelial cells. Studies have shown that non-pathogenic bacteria compete for these attachment sites on the intestinal epithelial cells, blocking the binding and subsequent entry of pathogenic and enteroinvasive bacteria into the epithelial cells. Moreover, as bacteria compete for nutrients in their environment and keep their collective habitat for the consumption of all resources, enteral microbiota can increase the competition for pathogenic bacteria for energy resources, increasing microbial proliferation. Thus, the bacteria of the intestinal microbiota can inhibit the growth of its competitors by producing antimicrobial substances known as bacteriocins (42).

Bacteria are essential for the early development of the immune system of the intestinal mucosa (41). The cells of the intestinal epithelium prevent threats from pathogens by signaling the innate immune system through specific receptors that recognize and bind molecules associated with bacteria, leading to production of a host immune response and release of protective peptides, cytokines, and leukocytes (39). The result can be a protective response to commensal bacteria, an inflammatory response to pathogenic organisms, or a trigger for host cell death (42).

Intestinal microbiota plays significant roles in both health and disease. Given its importance, there is a need to maintain an appropriate balance, which can be ensured by dietary supplementation of probiotics. Probiotics can confer health benefits to the host by modulation of the intestinal microbiota, microbiota stabilization after using antibiotics, alleviation of lactose intolerance symptoms, stimulation of immune response, relief from constipation, treatment of diarrhea, promotion of gastrointestinal colonization, resistance to pathogens, increased absorption of minerals, and vitamin production (41). Given the above, *L. lactis* acts as a probiotic, because it modulates the intestinal microflora and prevents the colonization of pathogenic microorganisms in the mucosa.

While assessing the survival of *L. lactis* in gastric and intestinal mucosa, the presence of this microorganism was observed in all sections of the GIT, however, alcohol reduced the proliferation of *L. lactis* in the organs.

Daniel and colleagues (43) investigated the temporal and spatial distribution of *L. lactis* subspecies *cremoris* in the GIT of mice using bioluminescence and found that it took about 90 minutes for *L. lactis* to reach the cecum and colon. After 4 and 6 hours of intragastric administration, the bacteria were localized along the cecum and colon, and after 24 hours, no microorganisms were detected in the intestines of mice. Moreover, these authors demonstrated that animals receiving *L. lactis* by gavage for 4 days showed no *L. lactis* in the GIT 24 hours after the last administration. This showed that externally administered *L. lactis* usually persists, but does not replicate actively or permanently to colonize the GIT.

In a study on axenic mice with intragastric administration of *L. lactis* subspecies *lactis* (10^6 CFU animal^{-1}), rapid growth of the bacteria was observed during the first 48 hours. The population of *L. lactis* was stable up to three months after the administration and showed no notable change during this period. By investigating the distribution of *L. lactis* in the different compartments of the lower digestive tract, the authors showed the presence of a large population

of *L. lactis* in the cecum. These data indicate that *L. lactis* adapts well to the physicochemical conditions of the digestive tract and has the ability to extract food resources to maintain a high level of its population (44).

To maintain the modulating activity of *L. lactis* in the GIT, continuous ingestion of a sufficient number of bacteria, for proliferation and colonization in the intestine, would be required. Intestinal colonization probability may increase after consumption of a greater number of viable cells.

Probiotics should be resistant to enzymes present in the oral cavity (e.g., lysozyme) and to digestion in the stomach conditions (e.g., exposure to low pH). As bacteria reach the intestinal tract, the bile in the duodenum of the small intestine is known to reduce their viability. Therefore, the ability to tolerate bile is another necessary characteristic of probiotics (45). Perrin *et al.* (46) have reported that the toxic effects of bile can be alleviated partially by adding a carbohydrate, which can be metabolized by certain strains of *Bifidobacterium*. The bile tolerance of some strains of *Lactococcus* varies according to the type of carbohydrate in the growth medium (47).

Intestinal adherence is important for many bacterial species for colonization of the GIT. It has been suggested that this adhesiveness is associated with the hydrophobicity of a strain (45). Growth conditions and stress can have a profound effect on the composition of lipid fatty acids and subsequently the hydrophobicity of the bacterial strain (48).

Kimoto-Nira *et al*. (45) evaluated the growth and survival of *L. lactis* subspecies *lactis* at low pH, grown with different carbohydrate sources, and observed that after exposure to pH 2.5 the number of viable cells reduced. They also investigated the effect of bile on *L. lactis*, showing that in the absence of bile, the bacterial growth was lower in the presence of fructose, xylose, galactose, and lactose; whereas in the presence of bile, the bacterial growth was significantly

lower on xylose and even lower on galactose. In the survival study, the number of colonies decreased in all carbon sources tested after incubation with bile, being lower in cells cultured on lactose. The authors investigated whether the type of carbohydrate affects the hydrophobicity and the fatty acid composition of the strain and observed that the strain grown on lactose had a high hydrophobicity.

The *in vitro* conditions of the simulated GIT are different from the *in vivo* conditions. For example, in the digestive tract, organisms administered orally are exposed to certain pH and digestive enzymes in the stomach, followed by exposure to bile in the intestine.

Studies have reported that alcohol can alter gastric pH, reduce intestinal motility, and disrupt bile production (49). Therefore, in this study, it was observed that although gastric and intestinal disorders caused by alcohol had been present, *L. lactis*, which was cultured in the presence of lactose and glucose, survived in the GIT with a decrease in bacterial proliferation.

Our findings point to the existence of direct and indirect effects of ethanol on the gastric and intestinal mucosa, limiting the adherence of the *Enterobacteriaceae* family bacteria. In addition, it was observed that *L. lactis*, acting as a probiotic, became more conducive to this environment, supporting colonization and diversification of *Enterobacteriaceae* in the GIT, leading to proliferation, especially of commensal species, which were able to survive and colonize the gastric and intestinal mucosa. The biotherapeutic modulation of the intestinal microbiota with this LAB appears to be a promising strategy to reduce alcohol-induced injuries in the GIT.

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Cristina de Carvalho Botelho for helpful discussions and critical reading of the manuscript and Dr. Anderson Miyoshi for attention and have provided the *L. lactis* subsp. *lactis* strain NCDO-2118.

Conflict of interest

The authors declare no conflict of interests, including personal or financial interests, direct or indirect connections, or any other situation that could raise questions of bias in either the reported work or the conclusions, implications, or opinions stated.

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Figures and figure legends

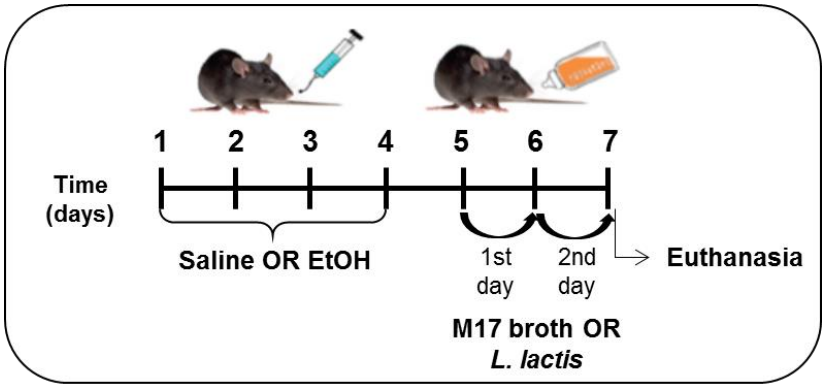


Figure 1. Experimental design. For four consecutive days, C57BL/6 female mice (n = 3) were given 50% (v/v) ethanol (EtOH) or saline solution by gavage. After 24 hours of last administration, the animals were administered *Lactococcus lactis* or M17 broth orally *ad libitum* for two consecutive days. Shortly after the second day of treatment, the animals were sacrificed and their stomach, small intestine, and large intestine (colon) were collected.

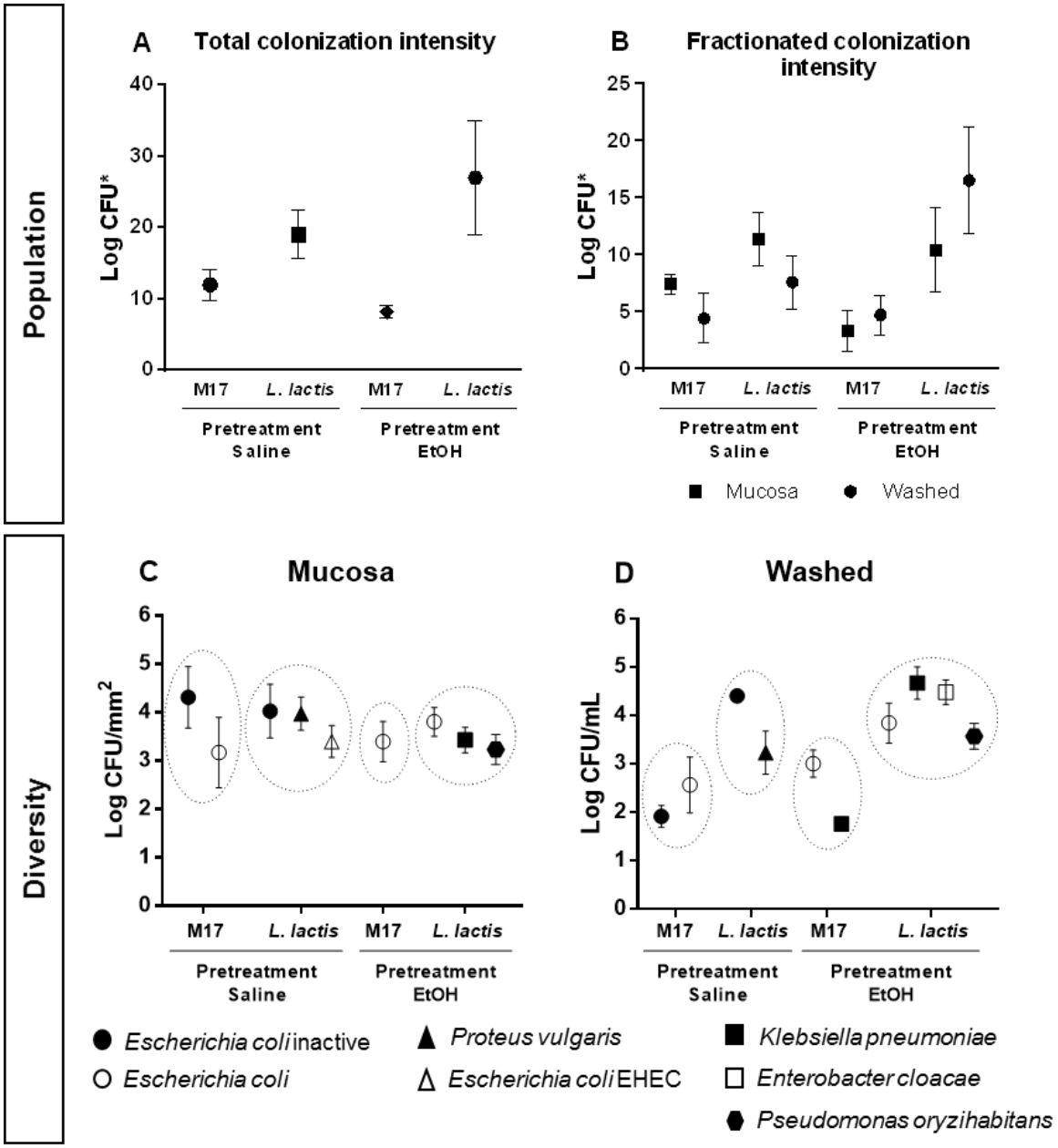


Figure 2. Effect of *Lactococcus lactis* on the abundance and diversity of *Enterobacteriaceae* species in the stomach, after ethanol (EtOH) administration. (A) Total colonization intensity. (B) Fractionated colonization intensity in the mucosa and washing samples. (C) Species diversity in the mucosal samples. (D) Species diversity in the washings (Washed). (A and B) Values are expressed as mean ± confidence interval for $\alpha = 0.05$. (C and D) Values are expressed as mean ± SEM, for three animals per group. * The results for mucosa are expressed as log CFU mm^{-2}, whereas the results for washings are expressed as log CFU mL^{-1}. M17, M17 broth.

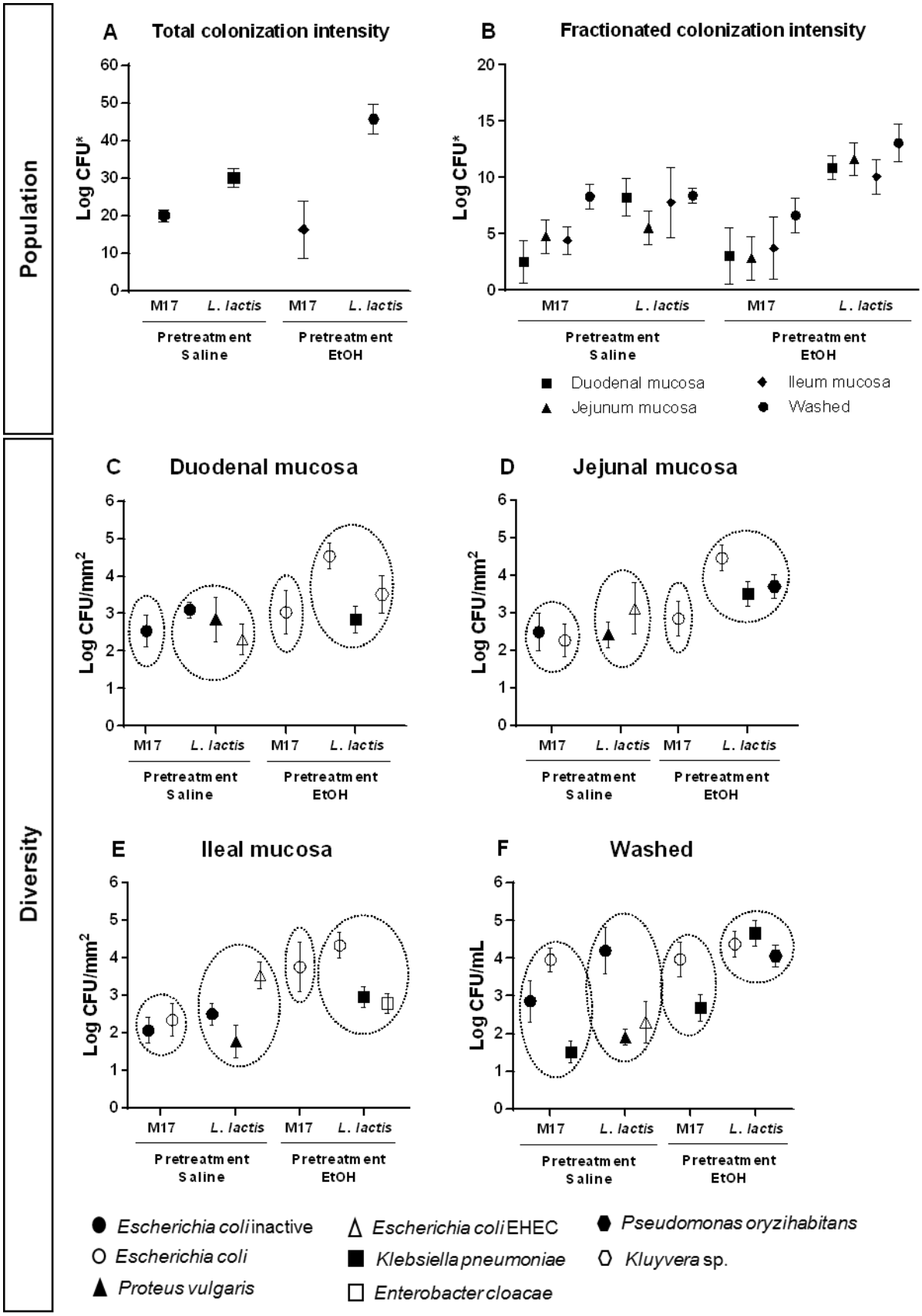

Population
Diversity
A
Total colonization intensity
Log CFU*
M17
L. lactis
Pretreatment Saline
Pretreatment EtOH
B
Fractionated colonization intensity
Duodenal mucosa
Jejunum mucosa
Ileum mucosa
Washed
C
Duodenal mucosa
Log CFU/mm2
D
Jejunal mucosa
E
Ileal mucosa
F
Washed
Log CFU/mL
Escherichia coli inactive
Escherichia coli
Proteus vulgaris
Escherichia coli EHEC
Klebsiella pneumoniae
Enterobacter cloacae
Pseudomonas oryzihabitans
Kluyvera sp.

Figure 3. Effect of *Lactococcus lactis* on the abundance and diversity of *Enterobacteriaceae* species in the small intestine, after ethanol (EtOH) administration. (A) Total colonization intensity. (B) Fractionated colonization intensity in the mucosa and washing samples. (C) Species diversity in the duodenal mucosa. (D) Species diversity in the jejunal mucosa. (E) Species diversity in the ileal mucosa. (E) Species diversity in the washings (Washed). (A and B) Values are expressed as mean ± confidence interval for $\alpha = 0.05$. (C, D, E, and F) Values are expressed as mean ± SEM, for three animals per group. * The results for mucosa are expressed as log CFU mm^{-2}, whereas the results for washings are expressed as log CFU mL^{-1}. M17, M17 broth.

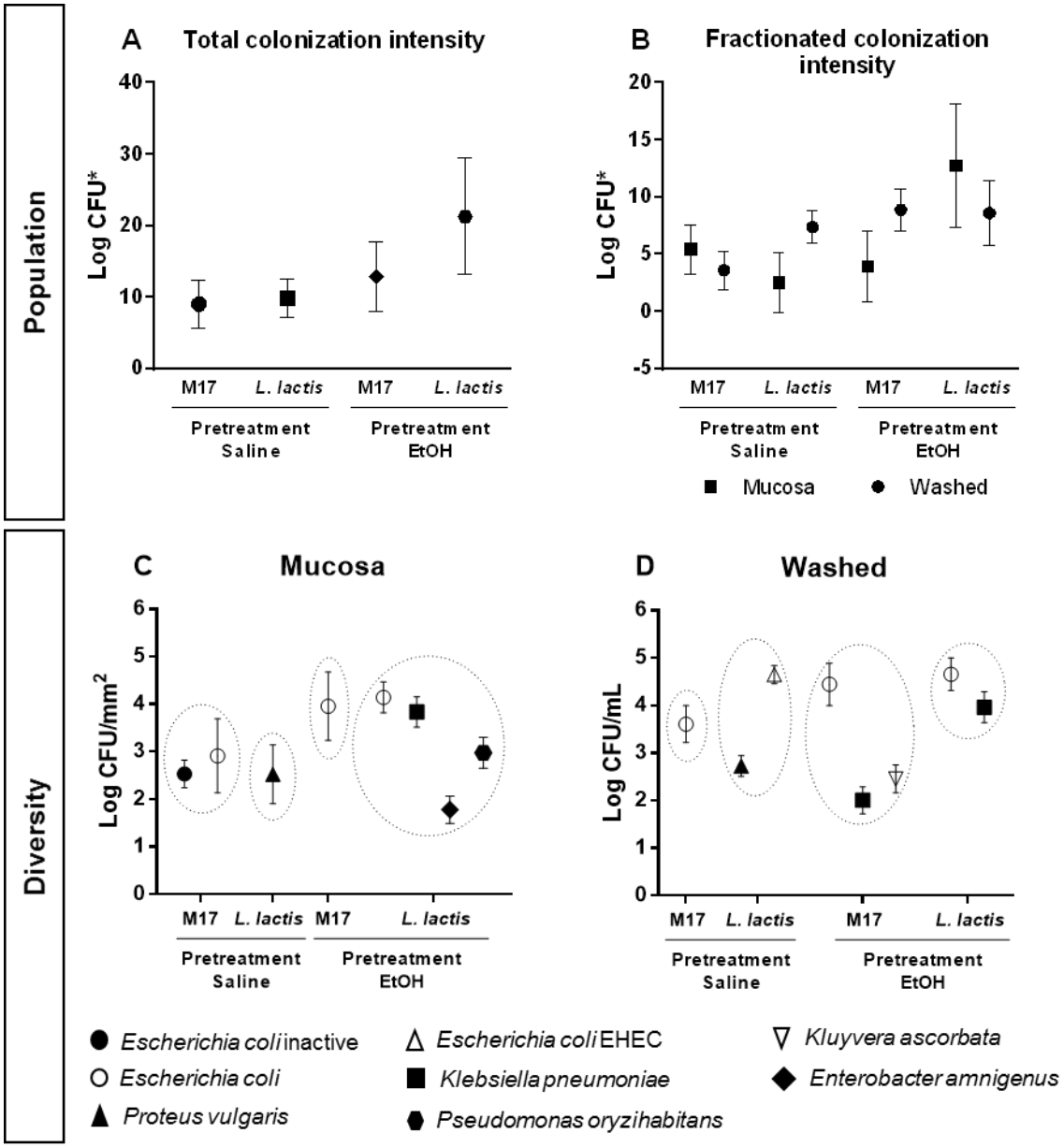


Figure 4. Effect of *Lactococcus lactis* on the abundance and diversity of *Enterobacteriaceae* species in the large intestine (colon), after ethanol (EtOH) administration. (A) Total colonization intensity. (B) Fractionated colonization intensity in the mucosa and washing samples. (C) Species diversity in the mucosal samples. (D) Species diversity in the washings (Washed). (A and B) Values are expressed as mean ± confidence interval for $\alpha = 0.05$. (C and D) Values are expressed as mean ± SEM, for three animals per group. * The results for mucosa are expressed as log CFU mm^{-2}, whereas the results for washings are expressed as log CFU mL^{-1}. M17, M17 broth.

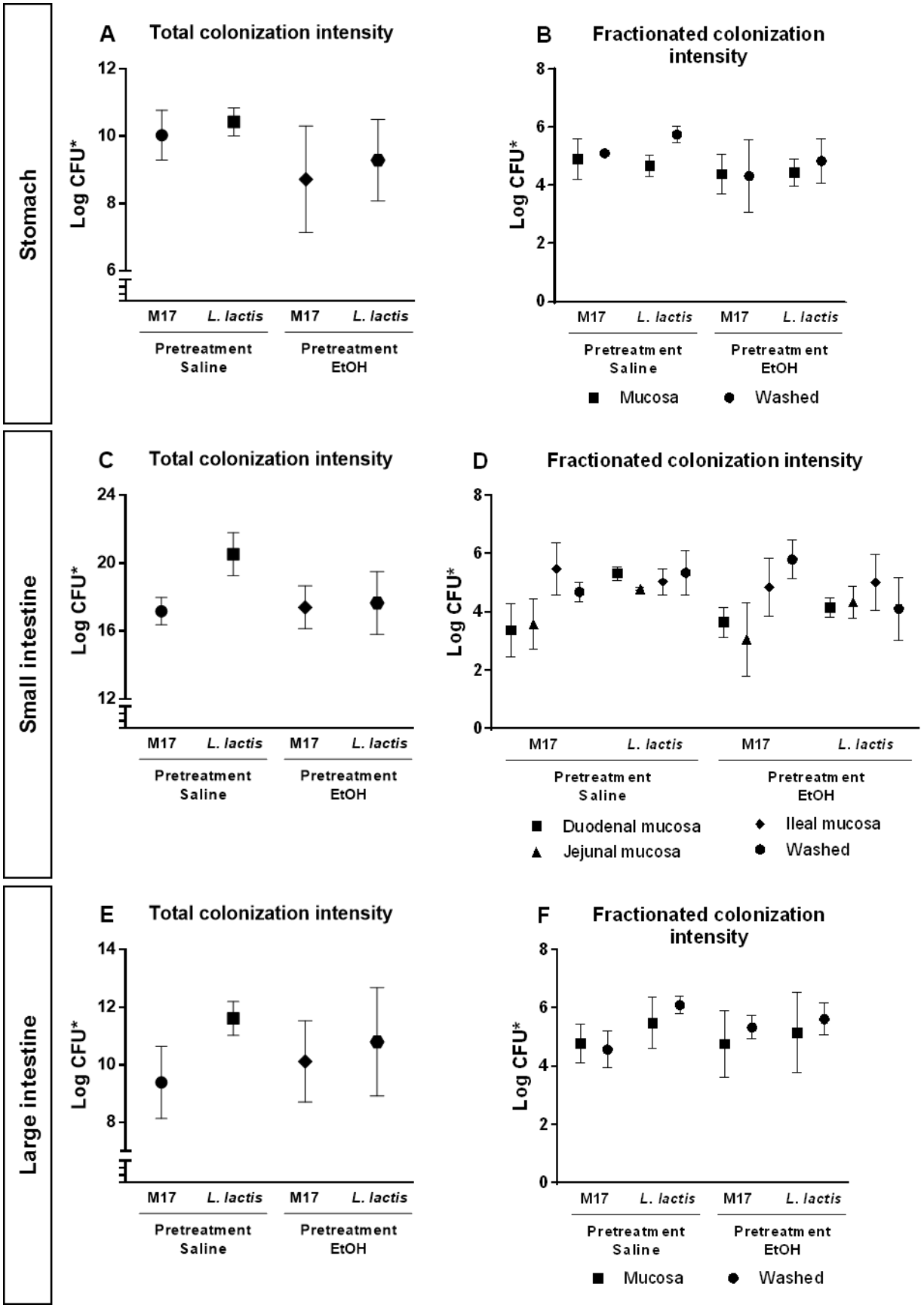

Stomach
A
Total colonization intensity
Log CFU*
12
10
8
6
M17
L. lactis
M17
L. lactis
Pretreatment
Saline
Pretreatment
EtOH
B
Fractionated colonization intensity
Log CFU*
8
6
4
2
0
M17
L. lactis
M17
L. lactis
Pretreatment
Saline
Pretreatment
EtOH
Mucosa
Washed
Small intestine
C
Total colonization intensity
Log CFU*
24
20
16
12
M17
L. lactis
M17
L. lactis
Pretreatment
Saline
Pretreatment
EtOH
D
Fractionated colonization intensity
Log CFU*
8
6
4
2
0
M17
L. lactis
M17
L. lactis
Pretreatment
Saline
Pretreatment
EtOH
Duodenal mucosa
Jejunal mucosa
Ileal mucosa
Washed
Large intestine
E
Total colonization intensity
Log CFU*
14
12
10
8
M17
L. lactis
M17
L. lactis
Pretreatment
Saline
Pretreatment
EtOH
F
Fractionated colonization intensity
Log CFU*
8
6
4
2
0
M17
L. lactis
M17
L. lactis
Pretreatment
Saline
Pretreatment
EtOH
Mucosa
Washed

Figure 5. Effect of *Lactococcus lactis* on the abundance of lactic acid bacteria in the gastrointestinal tract, after ethanol (EtOH) administration. (A) Total colonization intensity in the stomach. (B) Fractionated colonization intensity in the mucosa and washing samples from the stomach. (C) Total colonization intensity in the small intestine. (D) Fractionated colonization intensity in the mucosa and washing samples from the small intestine. (E) Total colonization intensity in the large intestine (colon). (F) Fractionated colonization intensity in the mucosa and washing samples from the large intestine (colon). Values are expressed as mean ± confidence interval for $\alpha = 0.05$, for three animals per group. * The results for mucosa are expressed as log CFU mm^{-2}, whereas the results for washings are expressed as log CFU mL^{-1}.

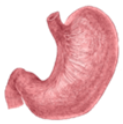
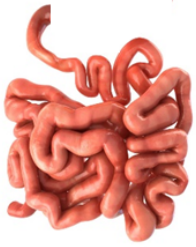
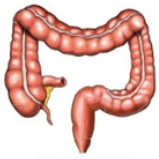
	EtOH	M17 broth	*L. lactis*
 Stomach	• Detachment of *Enterobacteriaceae* from the mucosa • Altered colonization intensity of LAB • ↓ presumptive *L. lactis* populations	• Selectivity for *Escherichia coli*	• ↑ abundance of *Enterobacteriaceae* • ↑ diversity of *Enterobacteriaceae*: *Enterobacter cloacae* *Pseudomonas oryzihabitans*
 Small intestine	• Detachment of *Enterobacteriaceae* from the mucosa • Altered colonization intensity of LAB • ↓ presumptive *L. lactis* populations	• Selectivity for *Escherichia coli*	• ↑ abundance of *Enterobacteriaceae* • ↑ diversity of *Enterobacteriaceae*: *Enterobacter cloacae* *Pseudomonas oryzihabitans* *Kluyvera* sp.
 Large intestine	• Altered colonization intensity of LAB • ↓ presumptive *L. lactis* populations		• ↑ abundance of *Enterobacteriaceae* • ↑ diversity of *Enterobacteriaceae*: *Pseudomonas oryzihabitans* *Enterobacter amnigenus*

Figure 6. Summary of changes observed in the gastrointestinal tract sections, after ethanol (EtOH) administration and *Lactococcus lactis* treatment. The data are presented for EtOH group, which was administered ethanol and treated with *L. lactis* or M17 broth, and were obtained in comparison to the saline control group. The M17 broth group was administered saline or ethanol and was treated with M17 broth. In turn, the data for the *L. lactis* group, which was previously administered ethanol and was treated with *L. lactis*, were obtained in comparison to the saline control group.

Table 1. Quantitative analysis of presumptive *Lactococcus lactis* in the gastrointestinal tract, after ethanol (EtOH) administration and *L. lactis* treatment.

Organ	*L. lactis*	EtOH - *L. lactis*	*p*-value
Stomach			
Washed	5,67 ± 0,02	3,74 ± 0,48	< 0,0001
Mucosa	4,59 ± 0,09	3,65 ± 0,05	< 0,01
Small intestine			
Washed	5,26 ± 0,13	3,75 ± 0,05	< 0,0001
Duodenal mucosa	5,33 ± 0,01	3,90 ± 0,03	< 0,0001
Jejunal mucosa	4,77 ± 0,01	3,21 ± 0,08	< 0,0001
Ileal mucosa	5,04 ± 0,03	4,69 ± 0,06	> 0,05
Large intestine			
Washed	6,11 ± 0,02	5,48 ± 0,01	< 0,05
Mucosa	5,50 ± 0,14	4,58 ± 0,17	< 0,01

Values are expressed as log CFU mL^{-1} for washed and log CFU mm^{-2} for mucosal samples. The data are reported as mean ± SEM, for three animals per group.

4 CONCLUSÕES

✓ Nas condições experimentais utilizadas neste estudo, conclui-se que o tratamento com *Lactococcus lactis* em animais que ingeriram etanol:

- reverteu o aumento no tamanho e peso gástricos.
- reduziu o grau de degeneração hepática, representado por uma menor presença de áreas vacuolizadas no interior dos hepatócitos.
- reverteu o aumento na produção de IgAs na mucosa gástrica.
- reverteu sinais clássicos de inflamação alérgica, através da redução dos níveis séricos de IgE total, dos níveis de IL-4 e IL-10 nas mucosas gástrica e intestinal, além de aumentar os níveis de IFN-γ gástrica.
- favoreceu as populações de *Enterobacteriaceae*.
- provocou maior diversidade de *Enterobacteriaceae* no estômago, intestino delgado e intestino grosso, evidenciando a presença de *Enterobacter cloacae*, *Pseudomonas oryzihabitans*, *Kluyvera* sp., *Enterobacter amnigenus* (habitantes normais do TGI) e *Klebsiella pneumoniae* (microrganismo patogênico).

✓ A ingestão de etanol pelos animais:

- provocou um desprendimento mais acentuado das Enterobacteriaceae da mucosa do estômago e intestino delgado.
- suprimiu em parte as populações de BAL em todo TGI.
- reduziu a proliferação de *L. lactis* nos órgãos do TGI.

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Broegelmann Research Laboratory
The Laboratory Building, 5th Floor, Room 5385
N-5021 Bergen, Norway

Telephone: +47-55 97 46 48/49
Fax: +47-55 97 58 17
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ANEXO B – Normas da revista do produto 2

AMERICAN SOCIETY FOR MICROBIOLOGY | Applied and Environmental Microbiology

August 2016, Instructions to Authors, pages 1–22

INSTRUCTIONS TO AUTHORS

SCOPE

Applied and Environmental Microbiology (AEM) publishes descriptions of all aspects of applied microbial research, basic research on microbial ecology, and research of a genetic and molecular nature that focuses on microbial topics of practical value. Research must address salient microbiological principles, fundamental microbial processes, or basic questions in applied or environmental microbiology. Topics that are considered include microbiology in relation to foods, agriculture, industry, biotechnology, public health, plants, and invertebrates and basic biological properties of bacteria, fungi, algae, protozoa, and other simple eukaryotic organisms as related to microbial ecology. Manuscripts should report new and significant findings that advance the understanding of microbiology and upon which other scientists may build. To best serve its readership, the journal must accept only those papers that are most significant to the field of applied and environmental microbiology. Thus, the editors will reject manuscripts that, while scientifically sound, represent only incremental extensions of other studies, are mainly confirmatory, or do not pursue a question in sufficient depth.

AEM publishes minireviews that provide forward-reaching assessments of topics of current relevance to the diverse sections of the journal. Additional information on minireviews can be found in a subsequent part of these Instructions.

AEM welcomes microbiome studies that address the microbiology and functions of natural or experimental systems. The nature of the microbiome study will determine in which section of the journal it will be published.

The **biodegradation** section describes novel microbial processes for alteration, removal, or utilization of environmental or anthropogenic chemicals.

Papers in the **biotechnology** section describe the use and modification of organisms in order to achieve socially beneficial objectives.

The **environmental microbiology** section covers manuscripts that focus on research related to microorganisms in the environment. This is distinct from the microbial ecology section, which focuses on ecological relationships, such as interactions among organisms, their structure and functional role in an ecosystem, and community-level studies. Thus, the environmental microbiology section features articles that focus on specific organisms in the environment, rather than a whole community, as well as those in which the study is not focused on implied or stated underlying ecological relationships.

The **enzymology and protein engineering** section covers a broad range of topics relative to microbial catalysis and includes papers describing (i) the structure and function of environmentally or industrially significant proteins and how they can be modified to achieve practical catalytic objectives and (ii) the enzymology or biosynthesis of fungal, algal, and bacterial metabolites or toxins of importance to the environment or to society.

Included in the **evolutionary and genomic microbiology** section are papers detailing newly described evolutionary processes and evolutionary relationships among microorganisms. Topics include genomic analysis of microorganisms and metagenomic investigation of microbiomes in the environment. (Meta)genome analyses that do not provide significant new insights into the microbiology of the system(s) under study will normally not be acceptable for publication in AEM.

The **food microbiology** section covers manuscripts dealing with all aspects of food microbiology, including microbial food pathogens, microbial ecology of foods, predictive food microbiology, food fermentations, food spoilage, probiotics, and prebiotics. Manuscripts detailing the occurrence of microbial toxins or microbial metabolites are suitable if the work includes significant information on the microbe and its toxin or metabolite production. This section also includes studies on the gastrointestinal tract microbiome as it relates to molecular toxicology, diet, and nutrition. Molecular assessments of food microbiomes should follow guidelines for the microbial ecology section.

The **genetics and molecular biology** section includes papers describing genetic organization, expression, mutation, and repair in organisms with environmental or practical significance.

Manuscripts for the **geomicrobiology** section must emphasize the role of microorganisms in geobiochemical processes in terrestrial or aquatic ecosystems, including subsurface, aquifer, and oceanic environments. Topics include mineralization, the use of inorganic ions in energy metabolism, and growth in extreme environments. Manuscripts focused on geological processes with only marginal links to microbiology will not qualify for AEM.

Invertebrate microbiology manuscripts should address interactions between invertebrates and microorganisms, ranging from commensalism and mutualism to parasitism and pathogenicity. Manuscripts describing work dealing with the metabolites or toxins from animal, plant, or insect cells or the physiology of such cells are not suitable for AEM unless the work concerns a microbial community or individual microorganisms.

New microbiological **methods** must provide novel avenues to address fundamental biological questions and will be considered for publication in AEM when accompanied by a demonstrated application. Descriptions of the application of previously described technologies, including the cloning, amplification, and expression of "foreign" genes, to a new genus or species of microbe will generally not be considered for independent publication. Manuscripts that describe the construction of engineered strains for innovative process application, development, or enhancement must present results to authenticate the utility, superiority, and uniqueness of such strains.

The **microbial ecology** section covers a wide range of topics on the ecology of microorganisms, including culture-independent

Instructions to Authors are updated throughout the year. The current version is available at http://journals.asm.org/t/49536.

molecular assessments that provide new insights into (i) the structure-function relationships of microorganisms, (ii) the impact of *in situ* conditions on community structure, or (iii) the effect of changes in microbial community composition on ecosystem function. Phylogenetic assessments that do not provide such insights will normally not be acceptable for publication in AEM.

The **physiology** section addresses questions about how organisms adapt to changes in their environment, including bioenergetics, stress, starvation, metabolic challenges, and responses to nutritional variation.

The **plant microbiology** section covers manuscripts dealing with all aspects of plant-microorganism interactions, including symbiotic and rhizosphere bacteria as well as phytopathogenic microorganisms.

The **public and environmental health microbiology** section is focused primarily on environmentally transmitted microorganisms that affect human health. Environmental health microbiology is a branch of public health concerned with the environmental occurrence of disease-causing microbes and with creating health-supportive environments. Microbes of a zoonotic nature or microbes transmitted through water, soil, or environmental surfaces are of special interest.

AEM is not specialized in the systematics of prokaryotes, but taxonomic papers that describe a new prokaryotic taxon are welcome when phylogenetic or genotypic data are accompanied by a significant amount of information that goes beyond the taxonomic description of the new taxon. Such additional information might include information on the novel ecological, physiological, biotechnological, or evolutionary features of the new taxa. Description of a new taxon should include an amount of information adequate to allow the new taxon to be validated and must include genus and species descriptions, which should be placed at the end of the Discussion section. Likewise, the new taxon must be deposited in two publically available culture collections that are in separate countries. Large data sets of comparative phenotypic and genotypic features (e.g., fatty acid compositions, substrate profiles, sequence similarities) or related species that might be of value for the taxonomic evaluation of the new taxon should normally be placed in supplemental material. The section of the journal in which such a paper will be placed will depend on the nature of the new taxon and the environment from which it was isolated.

ASM publishes a number of different journals covering various aspects of the field of microbiology. Each journal has a prescribed scope which must be considered in determining the most appropriate journal for each manuscript. The following guidelines may be of assistance.

(i) AEM will consider manuscripts describing properties of enzymes and proteins that are produced by either wild-type or genetically engineered microorganisms and that are significant or have potential significance in industrial or environmental settings. Studies dealing with basic biological phenomena of enzymes or proteins or in which enzymes have been used in investigations of basic biological functions are more appropriate for the *Journal of Bacteriology*.

(ii) AEM will consider papers which describe the use of antimicrobial agents as tools for elucidating aspects of applied and environmental microbiology. Other papers dealing with antimicrobial agents, including manuscripts dealing with the biosynthesis and metabolism of such agents, are more appropriate for *Antimicrobial Agents and Chemotherapy*.

(iii) AEM will consider manuscripts that concern bacteriophages or other viruses in relation to the environment, public health, or industrial microbiology. Papers that primarily concern attachment and intracellular replication of viruses, virus interactions with host metabolism, virus structure, or virus genomics are more appropriate for the *Journal of Virology*.

(iv) Manuscripts dealing with the immune system or with topics of basic medical interest or oral microbiology are more appropriate for *Infection and Immunity*. Reports of clinical investigations and environmental biology applied to hospitals should be submitted to the *Journal of Clinical Microbiology*.

(v) AEM and *mSphere*™ accept manuscripts on population dynamics and the ecology of eukaryotic microbes. Studies of microbial communities and of microbial populations with identified economic or ecological significance, e.g., plant pathogens or symbionts, are usually more appropriate for AEM.

(vi) Manuscripts dealing with the purification and characterization of enzymes or cloning of genes that have already been extensively described for other organisms will be considered for publication only if they offer experimentally supported new insights into the biological role, properties, or applications of these enzymes. Descriptions of genes or enzymes that differ only in minor ways from the prototypes are not suitable for AEM.

Questions about these guidelines may be directed to the editor in chief of the journal being considered.

If transfer to another ASM journal is recommended by an editor, the corresponding author will be contacted.

Note that a manuscript rejected by one ASM journal on scientific grounds or on the basis of its general suitability for publication is considered rejected by all other ASM journals.

EDITORIAL POLICY AND ETHICAL GUIDELINES

As a member of the Committee on Publication Ethics (COPE), ASM adheres to COPE's Best Practice Guidelines and expects authors to observe the high standards of publication ethics set out by COPE. ASM requirements for submitted manuscripts are consistent with the Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals, as last updated by the International Committee of Medical Journal Editors in December 2014 (http://www.icmje.org/).

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Use of Microbiological Information

The Council Policy Committee (CPC) of the American Society for Microbiology affirms the long-standing position of the Society that microbiologists will work for the proper and beneficent application of science and will call to the attention of the public or the appropriate authorities misuses of microbiology or of information derived from microbiology. ASM members are obligated to discourage any use of microbiology contrary to the welfare of humankind, including the use of microbes as biological weapons. Bioterrorism violates the fundamental principles expressed in the Code of Ethics of the Society and is abhorrent to ASM and its members.

ASM recognizes that there are valid concerns regarding the publication of information in scientific journals that could be put to inappropriate use as described in the CPC resolution mentioned above. Members of the ASM Journals Board will evaluate the rare manuscript that might raise such issues during the review process. However, as indicated elsewhere in these Instructions, research articles must contain sufficient detail, and material/information must be made available, to permit the work to be repeated by others. Supply of materials should be in accordance with laws and regulations governing the shipment, transfer, possession, and use of biological materials and must be for legitimate, bona fide research needs. We ask that authors pay particular attention to the NSAR Select Agent/Toxin list on the CDC website http://www.selectagents.gov/index.html and the U.S. Government Policy for Oversight of Life Sciences Dual Use Research of Concern (March 2012; http://www.phe.gov/s3/dualuse/Documents/us-policy-durc-032812.pdf).

Use of Human Subjects or Animals in Research

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Publishing Ethics

Authorship. ASM journals follow the criteria for authorship as outlined in the International Committee of Medical Journal Editors (ICMJE) Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals ("Defining the Role of Authors and Contributors"). Briefly, an author is one who makes a substantial contribution to the design, execution, and/or analysis and interpretation of experiments in addition to drafting, revising, and/or approving the initial submission and any subsequent versions of the article. All authors of a manuscript must have agreed to its submission and are responsible for appropriate portions of its content. Submission of a paper before all coauthors have read and approved it is considered an ethical violation.

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Culture deposition. AEM expects authors to deposit important strains in publicly accessible culture collections and to refer to the collections and strain numbers in the text. Since the authenticity of subcultures of culture collection specimens that are distributed by individuals cannot be ensured, authors should indicate laboratory strain designations and donor sources as well as original culture collection identification numbers.

Authentication of cell lines. Cell line misidentification or contamination can adversely impact the validity of research findings. Authors should describe the source along with the date and method used for authentication of any cell lines used in manuscripts submitted to this journal. Cell lines used less than 6 months after receipt from a cell bank that performs authentication do not require reauthentication, but the source and method of authentication should be reported in the Materials and Methods section.

Nucleotide and amino acid sequences. Newly determined nucleotide and/or amino acid sequence data must be deposited and GenBank/ENA/DDBJ accession numbers must be included in the manuscript no later than the modification stage of the review process. It is expected that the sequence data will be released to the public no later than the publication (online posting) date of the accepted manuscript. Authors are encouraged to comply with community metadata standards, such as the "Minimal Information about any (X) Sequence" (MIxS) checklist (http://gensc.org/projects/mixs-gsc-project/), when submitting to GenBank, ENA, or DDBJ. The accession numbers should be included in a separate paragraph with the lead-in "Accession number(s)" at the end of the Materials and Methods section. If conclusions in a manuscript are based on the analysis of sequences and a GenBank/ENA/DDBJ accession number is not provided at the time of the review, authors should provide the annotated sequence data as supplemental material not for publication.

It is expected that, when previously published sequence accession numbers are cited in a manuscript, the original citations (e.g., journal articles) will be included in the References section when possible or reasonable.

Authors are also expected to do elementary searches and comparisons of nucleotide and amino acid sequences against the se-

quences in standard databases (e.g., GenBank) immediately before manuscripts are submitted and again at the proof stage.

Analyses should specify the database, and the date of each analysis should be indicated as, e.g., January 2016. If relevant, the version of the software used should be specified.

See "Presentation of Nucleic Acid Sequences" for nucleic acid sequence formatting instructions.

The URLs of the databases mentioned above are as follows: DNA Data Bank of Japan (DDBJ), http://www.ddbj.nig.ac.jp/; European Nucleotide Archive (ENA), http://www.ebi.ac.uk/ena/; and GenBank, National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/nucleotide.

Proper use of locus tags as systematic identifiers for genes. To comply with recommendations from the International Nucleotide Sequence Database (INSD) Collaborators and to avoid conflicts in gene identification, researchers should implement the following two fundamental guidelines as standards for utilization of locus tags in genome analysis, annotation, submission, reporting, and publication. (i) Locus tag prefixes are systematic gene identifiers for all of the replicons of a genome and as such should be associated with a single genome project submission. (ii) New genome projects must be registered with the INSD, and new locus tag prefixes must be assigned in cooperation with the INSD to ensure that they conform to the agreed-upon criteria.

Structural determinations. Coordinates for new structures of macromolecules determined by X-ray crystallography or cryo-electron microscopy must be deposited in the Protein Data Bank and assigned identification codes must be included in the manuscript no later than the modification stage of the review process. It is expected that the coordinates will be released to the public no later than the publication (online posting) date of the accepted manuscript. Authors are encouraged to send coordinates with their original submission, however, so that reviewers can examine them along with the manuscript. The accession number(s) should be listed in a separate paragraph with the lead-in "Accession number(s)" at the end of the Materials and Methods section.

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Gene expression data. The entire set of supporting microarray, next-generation sequencing, or other high-throughput functional genomics data must be deposited in the appropriate public database (e.g., GEO, ArrayExpress, or CIBEX) and the assigned accession number(s) must be included in the manuscript no later than the modification stage of the review process. It is expected that the data will be released to the public no later than the publication (online posting) date of the accepted manuscript. Authors are encouraged to send the relevant data with their original submission, however, so that reviewers can examine them along with the manuscript. The accession number(s) should be listed in a separate paragraph with the lead-in "Accession number(s)" at the end of the Materials and Methods section.

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MycoBank. New scientific names of fungi along with key nomenclatural and descriptive material must be deposited in MycoBank (http://www.mycobank.org/) and the assigned accession number(s) must be included in the manuscript no later than the modification stage of the review process. It is expected that the data will be released to the public no later than the publication (online posting) date of the accepted manuscript. Authors are encouraged to send the relevant data with their original submission, however, so that reviewers can examine them along with the manuscript. The accession number(s) should be listed in a separate paragraph with the lead-in "Accession number(s)" at the end of the Materials and Methods section.

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5. **Cox CS, Brown BR, Smith JC.** J Gen Genet, in press.* {*Article title is optional; journal title is mandatory.*}
6. **Forman MS, Valsamakis A.** 2011. Specimen collection, transport, and processing: virology, p 1276–1288. *In* Versalovic J, Carroll KC, Jorgensen JH, Funke G, Landry ML, Warnock DW (ed), Manual of clinical microbiology, 10th ed, vol 2. ASM Press, Washington, DC.
7. **da Costa MS, Nobre MF, Rainey FA.** 2001. Genus I. Thermus Brock and Freeze 1969, 295,[AL] emend. Nobre, Trüper and da Costa 1996b, 605, p 404–414. *In* Boone DR, Castenholz RW, Garrity GM (ed), Bergey's manual of systematic bacteriology, 2nd ed, vol 1. Springer, New York, NY.

8. **Fitzgerald G, Shaw D.** *In* Waters AE (ed), Clinical microbiology, in press. EFH Publishing Co, Boston, MA.* {*Chapter title is optional.*}
9. **Green PN, Hood D, Dow CS.** 1984. Taxonomic status of some methylotrophic bacteria, p 251–254. *In* Crawford RL, Hanson RS (ed), Microbial growth on C_1 compounds. Proceedings of the 4th International Symposium. American Society for Microbiology, Washington, DC.
10. **Rotimi VO, Salako NO, Mohaddas EM, Philip LP.** 2005. Abstr 45th Intersci Conf Antimicrob Agents Chemother, abstr D-1658. {*Abstract title is optional.*}
11. **Smith D, Johnson C, Maier M, Maurer JJ.** 2005. Distribution of fimbrial, phage and plasmid associated virulence genes among poultry *Salmonella enterica* serovars, abstr P-038, p 445. Abstr 105th Gen Meet Am Soc Microbiol. American Society for Microbiology, Washington, DC. {*Abstract title is optional.*}
12. **García CO, Paira S, Burgos R, Molina J, Molina JF, Calvo C, Vega L, Jara LJ, García-Kutzbach A, Cuellar ML, Espinoza LR.** 1996. Detection of *Salmonella* DNA in synovial membrane and synovial fluid from Latin American patients using the polymerase chain reaction. Arthritis Rheum **39**(Suppl 9):S185. {*Meeting abstract published in journal supplement.*}
13. **O'Malley DR.** 1998. PhD thesis. University of California, Los Angeles, CA. {*Title is optional.*}
14. **Stratagene.** 2006. Yeast DNA isolation system: instruction manual. Stratagene, La Jolla, CA. {*Use the company name as the author if none is provided for a company publication.*}
15. **Odell JC.** April 1970. Process for batch culturing. US patent 484,363,770. {*Include the name of the patented item/process if possible; the patent number is mandatory.*}

*A reference to an in-press ASM publication should state the control number (e.g., AEM00123-16) if it is a journal article or the name of the publication if it is a book.

In some online journal articles, posting or revision dates may serve as the year of publication; a DOI (preferred) or URL is required for articles with nontraditional page numbers or electronic article identifiers.

Magalon A, Mendel RR. 15 June 2015, posting date. Biosynthesis and insertion of the molybdenum cofactor. EcoSal Plus 2015 doi:10.1128/ecosalplus.ESP-0006-2013.

Note: a posting or accession date is required for any online reference that is periodically updated or changed.

Citations of ASM Accepts manuscripts should look like the following example.

Wang GG, Pasillas MP, Kamps MP. 15 May 2006. Persistent transactivation by Meis1 replaces Hox function in myeloid leukemogenesis models: evidence for co-occupancy of Meis1-Pbx and Hox-Pbx complexes on promoters of leukemia-associated genes. Mol Cell Biol doi:10.1128/MCB.00586-06.

Other journals may use different styles for their publish-ahead-of-print manuscripts, but citation entries must include the following information: author name(s), posting date, title, journal title, and volume and page numbers and/or DOI. The following is an example:

Zhou FX, Merianos HJ, Brunger AT, Engelman DM. 13 February 2001. Polar residues drive association of polyleucine transmembrane helices. Proc Natl Acad Sci U S A doi:10.1073/pnas.041593698.

(ii) References cited in the text. References that should be cited in the text include the following:

- Unpublished data
- Manuscripts submitted for publication
- Unpublished conference presentations (e.g., a report or poster that has not appeared in published conference proceedings)
- Personal communications
- Patent applications and patents pending
- Computer software, databases, and websites

These references should be made parenthetically in the text as follows:

. . . similar results (R. B. Layton and C. C. Weathers, unpublished data).
. . . system was used (J. L. McInerney, A. F. Holden, and P. N. Brighton, submitted for publication).
. . . as described previously (M. G. Gordon and F. L. Rattner, presented at the Fourth Symposium on Food Microbiology, Overton, IL, 13 to 15 June 1989). {*For nonpublished abstracts and posters, etc.*}
. . . this new process (V. R. Smoll, 20 June 1999, Australian Patent Office). {*For non-U.S. patent applications, give the date of publication of the application.*}
. . . available in the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html).
. . . using ABC software (version 2.2; Department of Microbiology, State University [http://www.state.micro.edu]).

URLs for companies that produce any of the products mentioned in your study or for products being sold may not be included in the article. However, company URLs that permit access to scientific data related to the study or to shareware used in the study are permitted.

(iii) Citations in abstracts. Because the abstract must be able to stand apart from the article, references cited in it should be clear without recourse to the References section. Use an abbreviated form of citation, omitting the article title, as follows.

(P. S. Satheshkumar, A. S. Weisberg, and B. Moss, J Virol 87:10700–10709, 2013, doi:10.1128/JVI.01258-13)
(J. H. Coggin, Jr., p. 93–114, *in* D. O. Fleming and D. L. Hunt, ed., *Biological Safety. Principles and Practices*, 4th ed., 2006)
". . . in a recent report by D. A. Hopwood [mBio 4(5): e00612-13, 2013, doi:10.1128/mBio00612-13] . . . ."

This style should also be used for Addenda in Proof.

(iv) References related to supplemental material. If references must be cited in the supplemental material, list them in a **separate** References section within the supplemental material and cite them by those numbers; do not simply include citations of numbers from the reference list of the associated article. If the same reference(s) is to be cited in both the article itself and the supplemental material, then that reference would be listed in both References sections.

Short-Form Papers

AEM no longer considers papers in the short-form format. New submissions of research articles must be formatted as full-length papers.

Minireviews

Minireviews are brief (**limit of six printed pages exclusive of references**) biographical profiles, historical perspectives, or summaries of developments in fast-moving areas. They must be based on published articles; they may address any subject within the scope of AEM.

Minireviews may be either solicited or proffered by authors responding to a recognized need. Irrespective of origin, Minireviews are subject to review and should be submitted via the eJP online manuscript submission and peer review system. The cover letter should state whether the article was solicited and by whom.

Minireviews must have abstracts. Limit the abstract to 250 words or fewer. The body of the Minireview may have section headings and/or paragraph lead-ins.

Author bios. At the editor's invitation, corresponding authors of minireviews may submit a short biographical sketch and photo for each author for publication with the article. Biographical information should be submitted at the modification stage.

- The text limit is 150 words for each author and should include WHO you are (your name), WHERE you received your education, WHAT positions you have held and at WHICH institutions, WHERE you are now (your current institution), WHY you have this interest, and HOW LONG you have been in this field.
- The photo should be a black-and-white head shot of passport size. Photos will be reduced to approximately 1.125 inches wide by 1.375 inches high. Photos must meet the production criteria for regular figures and should be checked for production quality by using Rapid Inspector, provided at the following URL: http://rapidinspector.cadmus.com/RapidInspector/zmw/index.jsp.
- To submit, upload the text and photos with your modified manuscript in the submission and review system. Include the biographical text after the References section of your manuscript, in the same file. Upload the head shots in the submission system as a "Minireview Bio Photo"; **include the author's name or enough of it for identification in each photo's file name.**

Contact the scientific editor if you have questions about what to write. Contact the production editor if you have questions about submitting your files.

Meeting Reviews

Meeting Reviews are brief summaries of recent scientific meetings that cover topics within the scope of AEM. Reviews should be timely and focus on major themes, new developments, emerging trends, and significant unanswered questions presented and discussed at the meeting. Sufficient background should be provided to make the report useful to the general reader. The author must provide written assurance from the relevant individuals that permission to cite their presented material has been granted.

Meeting Reviews, which may be solicited or proffered by authors, are subject to editorial review and should be submitted via the eJP online manuscript submission and peer review system.

Commentaries

Commentaries are invited communications concerning topics relevant to the readership of AEM and are intended to engender discussion. Reviews of the literature, methods and other how-to papers, and responses targeted at a specific published paper are not appropriate. Commentaries are subject to review.

The length may not exceed four printed pages, and the format is like that of a Minireview (see above) except that the abstract is limited to 75 words.

Letters to the Editor

Letters to the Editor are intended only for comments on final, typeset articles published in the journal (not on accepted manuscripts posted online) and must cite published references to support the writer's argument.

Letters may be **no more than 500 words long and must be typed double-spaced**. Refer to a recently published Letter for correct formatting. Note that authors and affiliations are listed below the title.

All Letters to the Editor must be submitted electronically, and the manuscript type (Comment Letter) must be selected from the drop-down list in the submission form. The cover letter should state the volume and issue in which the article commented on was published, the title of the article, and the last name of the first author. In the Abstract section of the submission form, put "Not Applicable." Letters to the Editor do not have abstracts. The Letter must have a title, which must appear on the manuscript and on the submission form. Figures and tables should be kept to a minimum.

The Letter will be sent to the editor who handled the article in question. The letter may be sent for peer review. If the editor believes that publication is warranted, he/she will solicit a reply from the corresponding author of the article and make a recommendation to the editor in chief. Final approval for publication rests with the editor in chief.

Please note that some indexing/abstracting services do not include Letters to the Editor in their databases.

Errata

Errata provide a means of correcting errors that occurred during the writing, typing, editing, or publication (e.g., a misspelling, a dropped word or line, or mislabeling in a figure) of a published article. Submit Errata via the eJP online manuscript submission and peer review system (see "Submission, Review, and Publication Processes"). In the Abstract section of the submission form (a required field), put "Not Applicable." Upload the text of your Erratum as a Microsoft Word file. Please see a recent issue for correct formatting.

Author Corrections

Author Corrections provide a means of correcting errors of omission (e.g., author names or citations) and errors of a scientific nature that do not alter the overall basic results or conclusions of a published article (e.g., an incorrect unit of measurement or order of magnitude used throughout, contamination of one of numerous cultures, or misidentification of a mutant strain, causing erroneous data for only a [noncritical] portion of the study). Note that the addition of new data is not permitted.

For corrections of a scientific nature or issues involving authorship, including contributions and use or ownership of data and/or materials, all disputing parties must agree, in writing, to publication of the Correction. For omission of an author's name, letters must be signed by the authors of the article and the author whose name was omitted. The editor who handled the article will be consulted if necessary.

Submit an Author Correction via the eJP online manuscript submission and peer review system (see "Submission, Review, and Publication Processes"). Select Author Correction as the manuscript type. In the Abstract section of the submission form (a required field), put "Not Applicable." Upload the text of your Author Correction as a Microsoft Word file. Please see a recent issue for correct formatting. Signed letters of agreement must be supplied as supplemental material not for publication (scanned PDF files).

Retractions

Retractions are reserved for major errors or breaches of ethics that, for example, may call into question the source of the data or the validity of the results and conclusions of an article. Submit Retractions via the eJP online manuscript submission and peer review system (see "Submission, Review, and Publication Processes"). In the Abstract section of the submission form (a required field), put "Not Applicable." Upload the text of your Retraction as a Microsoft Word file. Letters of agreement signed by all of the authors must be supplied as supplemental material not for publication (scanned PDF files). The Retraction will be assigned to the editor in chief of the journal, and the editor who handled the paper and the chairperson of the ASM Journals Board will be consulted. If all parties agree to the publication and content of the Retraction, it will be sent to the Journals Department for publication.

CrossMark

ASM has implemented CrossMark. CrossMark is a multipublisher initiative to provide a standard way for readers to locate the current version of an article. Clicking on the CrossMark logo will indicate whether an article is current or whether updates have been published. Additional information about CrossMark can be found on CrossMark's website and on ASM's CrossMark policy page.

ILLUSTRATIONS AND TABLES

Illustrations

Image manipulation. Digital images submitted for publication may be inspected by ASM production specialists for any manipulations or electronic enhancements that may be considered to be the result of scientific misconduct based on the guidelines provided below. Any images/data found to contain manipulations of concern will be referred to the editor in chief, and authors may then be requested to provide their primary data for comparison with the submitted image file. Investigation of the concerns may delay publication and may result in revocation of acceptance and/or additional action by ASM.

Linear adjustments to contrast, brightness, and/or color are generally acceptable, as long as the measures taken are necessary to view elements that are already present in the data and the adjustments are applied to the entire image and not just specific areas. Unacceptable adjustments to images include, but are not limited to, the removal or deletion, concealment, duplication (copying and pasting), addition, selective enhancement, or repositioning of elements within the image.

Nonlinear adjustments made to images, such as changes to gamma settings, should be fully disclosed in the figure legends at the time of submission. In addition, images created by compiling multiple files, including noncontiguous portions of the same image, should clearly distinguish that these multiple files are not a single image. This can be done by "tooling," or inserting thin lines, between the individual images.

File types and formats. Illustrations may be continuous-tone images, line drawings, or composites. Color graphics may be submitted. Suggestions about how to ensure accurate color reproduction are given below.

On initial submission, figures may be uploaded as individual PDF files or combined and uploaded as a single PDF file. Place each legend in the text file, as well as on the same page with the corresponding figure to assist review. At the modification stage, production-quality digital files must be provided. Because the legends will be copyedited and typeset for final publication, they should appear within the main text, after the References section, and should not be included as part of the figure itself at this stage. All graphics submitted with modified manuscripts must be bitmap, grayscale, or in the RGB (preferred) or CMYK color mode. See "Color illustrations." Halftone images (those with various densities or shades) must be grayscale, not bitmap. AEM accepts TIFF or EPS files but discourages the use of PowerPoint for either black-and-white or color images.

For instructions on creating acceptable EPS and TIFF files,

refer to the Cadmus digital art website, http://art.cadmus.com/da/index.jsp. PowerPoint requires users to pay close attention to the fonts used in their images (see the section on fonts below). If instructions for fonts are not followed exactly, images prepared for publication are subject to missing characters, improperly converted characters, or shifting/obscuring of elements or text in the figure. For proper font use in PowerPoint images, refer to the Cadmus digital art website, http://art.cadmus.com/da/instructions/ppt_disclaimer.jsp. Note that, due to page composition system requirements, you must verify that your PowerPoint files can be converted to PDF without any errors.

We strongly recommend that before returning their modified manuscripts, authors check the acceptability of their digital images for production by running their files through Rapid Inspector, a tool provided at the following URL: http://rapidinspector.cadmus.com/RapidInspector/zmw/index.jsp. Rapid Inspector is an easy-to-use, Web-based application that identifies file characteristics that may render the image unusable for production. Please note when using Rapid Inspector to check PowerPoint files that there is a known bug in the application that can occasionally fail PowerPoint Presentation (.pptx) files, even though the files meet all required production criteria. If you experience this bug, the issue can be corrected by saving the PowerPoint files as an older version, PowerPoint 97-2004 Presentation (.ppt), during the Save As process (use the drop-down format menu and select this format). Once you save your files as .ppt, they will pass Rapid Inspector if all required production criteria have been met.

If you have additional questions about using the Rapid Inspector preflighting tool, please send an e-mail inquiry to helpdesk.digitalartsupport@cenveo.com.

Minimum resolution. It is extremely important that a high enough resolution is used. All separate images that you import into a figure file must be at the correct resolution before they are placed. (For instance, placing a 72-dpi image in a 300-dpi EPS file will not result in the placed image meeting the minimum requirements for file resolution.) Note, however, that the higher the resolution, the larger the file and the longer the upload time. Publication quality will not be improved by using a resolution higher than the minimum. Minimum resolutions are as follows:

- 300 dpi for grayscale and color
- 600 dpi for combination art (lettering and images)
- 1,200 dpi for line art

Size. All graphics **should be submitted at their intended publication size;** that is, the image uploaded should be 100% of its print dimensions so that no reduction or enlargement is necessary. Resolution must be at the required level at the submitted size. Include only the significant portion of an illustration. White space must be cropped from the image, and excess space between panel labels and the image must be eliminated.

- Maximum width for a 1-column figure: 20.6 picas (ca. 8.7 cm)
- Maximum width for a 2-column figure: 42 picas (ca. 17.8 cm)
- Minimum width for a 2-column figure: 26 picas (11.1 cm)
- Maximum height for a standard figure: 54.7 picas (ca. 23.2 cm)
- Maximum height for an oversized figure (no running title): 57.4 picas (ca. 24.3 cm)

Contrast. Illustrations must contain sufficient contrast to be viewed easily on a monitor or on the printed page.

Labeling and assembly. All final lettering and labeling must be incorporated into the figures. On initial submission, illustrations should be provided as PDF files, with the legends in the text file and with a legend beneath each image to assist review. At the modification stage, production-quality digital figure files (without legends) must be provided. Put the figure number well outside the boundaries of the image itself. (Numbering may need to be changed at the copyediting stage.) Each figure must be uploaded as a separate file, and any multipanel figures must be assembled into one file; i.e., rather than uploading a separate file for each panel in a figure, assemble all panels in one piece and supply them as one file.

Fonts. To avoid font problems, set all type in one of the following fonts: Arial, Helvetica, Times Roman, European PI, Mathematical PI, or Symbol. Courier may be used but should be limited to nucleotide or amino acid sequences, where a nonproportional (monospace) font is required. All fonts other than these must be converted to paths (or outlines) in the application with which they were created.

Color illustrations. All figures submitted in color will be processed as color. Adherence to the following guidelines will help to ensure color reproduction that is as accurate as possible.

The final online version is considered the version of record for AEM and all other ASM journals. To maximize online reproduction, color illustrations should be supplied in the RGB color mode as either (i) RGB TIFF images with a resolution of at least 300 pixels per inch (raster files, consisting of pixels) or (ii) Illustrator-compatible EPS files with RGB color elements (vector files, consisting of lines, fonts, fills, and images). CMYK files are also accepted. Other than in color space, CMYK files must meet the same production criteria as RGB files. The RGB color space is the native color space of computer monitors and of most of the equipment and software used to capture scientific data, and it can display a wider range of colors (especially bright fluorescent hues) than the CMYK (cyan, magenta, yellow, black) color space used by print devices that put ink (or toner) on paper. For reprints, ASM's print provider will automatically create CMYK versions of color illustrations from the supplied RGB versions. Color in the reprints may not match that in the online journal of record because of the smaller range of colors capable of being reproduced by CMYK inks on a printing press. For additional information on RGB versus CMYK color, refer to the Cadmus digital art site, http://art.cadmus.com/da/guidelines_rgb.jsp.

Drawings

Submit graphs, charts, complicated chemical or mathematical formulas, diagrams, and other drawings as finished products not requiring additional artwork or typesetting. All elements, including letters, numbers, and symbols, must be easily readable, and both axes of a graph must be labeled.

When creating line art, please use the following guidelines:

(i) **All art must be submitted at its intended publication size.** For acceptable dimensions, see "Size" above.

(ii) **Avoid using screens (i.e., shading) in line art.** It can be difficult and time-consuming to reproduce these images without moiré patterns. Various pattern backgrounds are preferable to screens as long as the patterns are not imported from another application. If you must use images containing screens,

(a) Generate the image at line screens of 85 lines per inch or less.

(b) When applying multiple shades of gray, differentiate the gray levels by at least 20%.

(c) Never use levels of gray below 5% or above 95% as they are likely to fade out or become totally black when output.

(iii) Use thick, solid lines that are no finer than 1 point in thickness.

(iv) No type should be smaller than 6 points at the final publication size.

(v) Avoid layering type directly over shaded or textured areas.

(vi) Avoid the use of reversed type (white lettering on a black background).

(vii) Avoid heavy letters, which tend to close up, and unusual symbols, which the printer may not be able to reproduce in the legend.

(viii) If colors are used, avoid using similar shades of the same color and avoid very light colors.

In figure ordinate and abscissa scales (as well as table column headings), avoid the ambiguous use of numbers with exponents. Usually, it is preferable to use the Système International d'Unités (SI) symbols (μ for 10^{-6}, m for 10^{-3}, k for 10^{3}, and M for 10^{6}, etc.). Thus, representation of 20,000 cpm on a figure ordinate should be made by the number 20 accompanied by the label kcpm. A complete listing of SI symbols can be found in the International Union of Pure and Applied Chemistry (IUPAC) publication *Quantities, Units and Symbols in Physical Chemistry*, 3rd ed. (RSC Publishing, Cambridge, United Kingdom, 2007), and at http://www.nist.gov/pml/pubs/sp811/.

When powers of 10 must be used, the journal requires that the exponent power be associated with the number shown. In representing 20,000 cells per ml, the numeral on the ordinate should be "2" and the label should be "10^{4} cells per ml" (not "cells per ml × 10^{-4}"). Likewise, an enzyme activity of 0.06 U/ml might be shown as 6 accompanied by the label 10^{-2} U/ml. The preferred designation is 60 mU/ml (milliunits per milliliter).

Presentation of Nucleic Acid Sequences

Long nucleic acid sequences must be presented as figures in the following format to conserve space. Print the sequence in lines of approximately 100 to 120 nucleotides in a nonproportional (monospace) font that is easily legible when published with a line length of 6 inches (ca. 15.2 cm). If possible, lines of nucleic acid sequence should be further subdivided into blocks of 10 or 20 nucleotides by spaces within the sequence or by marks above it. Uppercase and lowercase letters may be used to designate the exon-intron structure or transcribed regions, etc., if the lowercase letters remain legible at a 6-inch (ca. 15.2-cm) line length. Number the sequence line by line; place numerals representing the first base of each line to the left of the lines. Minimize spacing between lines of sequence, leaving room only for annotation of the sequence. Annotation may include boldface, underlining, brackets, and boxes, etc. Encoded amino acid sequences may be presented, if necessary, immediately above or below the first nucleotide of each codon, by using the single-letter amino acid symbols. Comparisons of multiple nucleic acid sequences should conform as closely as possible to the same format.

Figure Legends

On initial submission, each legend should be placed in the text file *and* be incorporated into the image file beneath the figure to assist review.

Legends should provide enough information so that the figure is understandable without frequent reference to the text. However, detailed experimental methods must be described in the Materials and Methods section, not in a figure legend. A method that is unique to one of several experiments may be reported in a legend only if the discussion is very brief (one or two sentences). Define all symbols used in the figure, and define all abbreviations that are not used in the text.

Tables

Tables that contain artwork, chemical structures, or shading must be submitted as illustrations in an acceptable format at the modification stage. The preferred format for regular tables is Microsoft Word; however, WordPerfect and Acrobat PDF are also acceptable. Note that a straight Excel file is not currently an acceptable format. Excel files must be either embedded in a Word or WordPerfect document or converted to PDF before being uploaded.

Tables should be formatted as follows. Arrange the data so that **columns of like material read down, not across.** The headings should be sufficiently clear so that the meaning of the data is understandable without reference to the text. See the "Abbreviations" section of these Instructions for those that should be used in tables. Explanatory footnotes are acceptable, but more-extensive table "legends" are not. Footnotes should not include detailed descriptions of the experiment. Tables must include enough information to warrant table format; those with fewer than six pieces of data will be incorporated into the text by the copy editor. Table 1 is an example of a well-constructed table.

TABLE 1 Distribution of protein and ATPase in fractions of dialyzed membranes[a]

Membrane	Fraction	ATPase	
		U/mg of protein	Total U
Control	Depleted membrane	0.036	2.3
	Concentrated supernatant	0.134	4.82
EI treated	Depleted membrane	0.034	1.98
	Concentrated supernatant	0.11	4.6

[a] Specific activities of ATPase of nondepleted membranes from control and treated bacteria were 0.21 and 0.20, respectively.

Cover Photographs and Drawings

AEM publishes photographs and drawings on the front cover. Invitations are issued to authors whose manuscripts are returned for modification or whose manuscripts have been accepted for publication in AEM; material should be related to the work presented in the AEM manuscript. Unsolicited photos can be submitted in hard-copy format (two copies) only; if an unsolicited photo is chosen for the cover, the author will be asked to submit digital files. No material submitted for consideration will be returned to the author. Authors will be notified only if their cover art is selected. Copyright for the chosen material must be transferred to ASM. A short description of the cover material will be included at the end of the table of contents. Technical specifications for submission and comments on potential illustrations can be obtained from the cover editor, Patrick D. Schloss (pschloss@umich.edu).

NOMENCLATURE

Chemical and Biochemical Nomenclature

The recognized authority for the names of chemical compounds is *Chemical Abstracts* (CAS; http://www.cas.org/) and its indexes. *The Merck Index Online* (https://www.rsc.org/merck-index) is also an excellent source. For biochemical terminology, including abbreviations and symbols, consult *Biochemical Nomenclature and Related Documents* (Portland Press, London, United Kingdom, 1992), available at http://www.chem.qmul.ac.uk/iupac/bibliog/white.html, and the instructions to authors of the *Journal of Biological Chemistry* and the *Archives of Biochemistry and Biophysics*.

Do not express molecular weight in daltons; molecular weight is a unitless ratio. Molecular mass is expressed in daltons.

For enzymes, use the recommended (trivial) name assigned by the Nomenclature Committee of the International Union of Biochemistry (IUB) as described in *Enzyme Nomenclature* (Academic Press, Inc., New York, NY, 1992) and its supplements and at http://www.chem.qmul.ac.uk/iubmb/enzyme/. If a nonrecommended name is used, place the proper (trivial) name in parentheses at first use in the abstract and text. Use the EC number when one has been assigned. Authors of papers describing enzymological studies should review the standards of the STRENDA Commission for information required for adequate description of experimental conditions and for reporting enzyme activity data (http://www.beilstein-institut.de/en/projects/strenda/guidelines).

Nomenclature of Microorganisms

Binary names, consisting of a generic name and a specific epithet (e.g., *Escherichia coli*), must be used for all microorganisms. Names of categories at or above the genus level may be used alone, but specific and subspecific epithets may not. A specific epithet must be preceded by a generic name, written out in full the first time it is used in a paper. Thereafter, the generic name should be abbreviated to the initial capital letter (e.g., *E. coli*), provided there can be no confusion with other genera used in the paper. Names of all bacterial taxa (kingdoms, phyla, classes, orders, families, genera, species, and subspecies) are printed in italics and should be italicized in the manuscript; strain designations and numbers are not. Vernacular (common) names should be in lowercase roman type (e.g., streptococcus, brucella). For *Salmonella*, genus, species, and subspecies names should be rendered in standard form: *Salmonella enterica* at first use, *S. enterica* thereafter; *Salmonella enterica* subsp. *arizonae* at first use, *S. enterica* subsp. *arizonae* thereafter. Names of serovars should be in roman type with the first letter capitalized: *Salmonella enterica* serovar Typhimurium. After the first use, the serovar may also be given without a species name: *Salmonella* Typhimurium, *S.* Typhimurium, or *Salmonella* serovar Typhimurium. For other information regarding serovar designations, see *Antigenic Formulae of the Salmonella Serovars*, 9th ed. (P. A. D. Grimont and F.-X. Weill, WHO Collaborating Centre for Reference and Research on *Salmonella*, Institut Pasteur, Paris, France, 2007; see http://www.scacm.org/free/Antigenic%20Formulae%20of%20the%20Salmonella%20Serovars%202007%209th%20edition.pdf). For a summary of the current standards for *Salmonella* nomenclature and the Kaufmann-White criteria, see the article by Brenner et al. (J Clin Microbiol **38**:2465–2467, 2000), the opinion of the Judicial Commission of the International Committee on Systematics of Prokaryotes (Int J Syst Evol Microbiol **55**:519–520, 2005), and the article by Tindall et al. (Int J Syst Evol Microbiol **55**:521–524, 2005).

The spelling of bacterial names should follow the *Approved Lists of Bacterial Names (Amended) & Index of the Bacterial and Yeast Nomenclatural Changes* (V. B. D. Skerman et al., ed., American Society for Microbiology, Washington, DC, 1989) and the validation lists and notification lists published in the *International Journal of Systematic and Evolutionary Microbiology* (formerly the *International Journal of Systematic Bacteriology*) since January 1989. In addition, two sites on the World Wide Web list current approved bacterial names: Prokaryotic Nomenclature Up-to-Date (http://www.dsmz.de/bacterial-diversity/prokaryotic-nomenclature-up-to-date.html) and the List of Prokaryotic Names with Standing in Nomenclature (http://www.bacterio.net/). If there is reason to use a name that does not have standing in nomenclature, the name should be enclosed in quotation marks in the title and at its first use in the abstract and the text and an appropriate statement concerning the nomenclatural status of the name should be made in the text. "*Candidatus*" species should always be set in quotation marks.

For guidelines regarding new names and descriptions of new genera and species, see the articles by Tindall (Int J Syst Bacteriol **49**:1309–1312, 1999) and Stackebrandt et al. (Int J Syst Evol Microbiol **52**:1043–1047, 2002). To validate new names and/or combinations, authors must submit three copies of

their published article to the *International Journal of Systematic and Evolutionary Microbiology*.

It is recommended that a strain be deposited in at least two recognized culture collections in different countries when that strain is necessary for the description of a new taxon (Int J Syst Evol Microbiol **50:**2239–2244, 2000).

Since the classification of fungi is not complete, it is the responsibility of the author to determine the accepted binomial for a given organism. Sources for these names include *The Yeasts: a Taxonomic Study*, 5th ed. (C. P. Kurtzman, J. W. Fell, and T. Boekhout, ed., Elsevier Science, Amsterdam, Netherlands, 2011), and *Dictionary of the Fungi*, 10th ed. (P. M. Kirk, P. F. Cannon, D. W. Minter, and J. A. Stalpers, ed., CABI International, Wallingford, Oxfordshire, United Kingdom, 2008); see also http://www.speciesfungorum.org/Names/Fundic.asp.

Names used for viruses should be those approved by the International Committee on Taxonomy of Viruses (ICTV) and reported on the ICTV Virus Taxonomy website (http://www.ictvonline.org/index.asp). In addition, the recommendations of the ICTV regarding the use of species names should generally be followed: when the entire species is discussed as a taxonomic entity, the species name, as with other taxa, is italic and has the first letter and any proper nouns capitalized (e.g., *Tobacco mosaic virus*, *Murray Valley encephalitis virus*). When the behavior or manipulation of individual viruses is discussed, the vernacular (e.g., tobacco mosaic virus, Murray Valley encephalitis virus) should be used. If desired, synonyms may be added parenthetically when the name is first mentioned. Approved generic (or group) and family names may also be used.

Microorganisms, viruses, and plasmids should be given designations consisting of letters and serial numbers. It is generally advisable to include a worker's initials or a descriptive symbol of locale or laboratory, etc., in the designation. Each new strain, mutant, isolate, or derivative should be given a new (serial) designation. This designation should be distinct from those of the genotype and phenotype, and genotypic and phenotypic symbols should not be included. Plasmids are named with a lowercase "p" followed by the designation in uppercase letters and numbers. To avoid the use of the same designation as that of a widely used strain or plasmid, check the designation against a publication database such as Medline.

For submissions on the topic of probiotics, the Food and Agriculture Organization and World Health Organization (FAO/WHO) definition must be used: "live microorganisms, which when administered in adequate amounts, confer a health benefit on the host." To avoid any misrepresentation of how this term should be applied, authors are encouraged to read the FAO/WHO Guidelines published in 2002 (ftp://ftp.fao.org/es/esn/food/wgreport2.pdf).

Genetic Nomenclature

To facilitate accurate communication, **it is important that standard genetic nomenclature be used whenever possible and that deviations or proposals for new naming systems be endorsed by an appropriate authoritative body**. Review and/or publication of submitted manuscripts that contain new or nonstandard nomenclature may be delayed by the editor or the Journals Department so that they may be reviewed.

Bacteria. The genetic properties of bacteria are described in terms of phenotypes and genotypes. The phenotype describes the observable properties of an organism. The genotype refers to the genetic constitution of an organism, usually in reference to some standard wild type. The guidelines that follow are based on the recommendations of Demerec et al. (Genetics **54:**61–76, 1966).

(i) Phenotypic designations must be used when mutant loci have not been identified or mapped. They can also be used to identify the protein product of a gene, e.g., the OmpA protein. Phenotypic designations generally consist of three-letter symbols; these are not italicized, and the first letter of the symbol is capitalized. It is preferable to use Roman or Arabic numerals (instead of letters) to identify a series of related phenotypes. Thus, a series of nucleic acid polymerase mutants might be designated Pol1, Pol2, and Pol3, etc. Wild-type characteristics can be designated with a superscript plus (Pol^+), and, when necessary for clarity, negative superscripts (Pol^-) can be used to designate mutant characteristics. Lowercase superscript letters may be used to further delineate phenotypes (e.g., Str^r for streptomycin resistance). Phenotypic designations should be defined.

(ii) Genotypic designations are also indicated by three-letter locus symbols. In contrast to phenotypic designations, these are lowercase italic (e.g., *ara his rps*). If several loci govern related functions, these are distinguished by italicized capital letters following the locus symbol (e.g., *araA araB araC*). Promoter, terminator, and operator sites should be indicated as described by Bachmann and Low (Microbiol Rev **44:**1–56, 1980), e.g., *lacZp*, *lacAt*, and *lacZo*.

(iii) Wild-type alleles are indicated with a superscript plus (ara^+ his^+). A superscript minus is not used to indicate a mutant locus; thus, one refers to an *ara* mutant rather than an ara^- strain.

(iv) Mutation sites are designated by placing serial isolation numbers (allele numbers) after the locus symbol (e.g., *araA1 araA2*). If it is not known in which of several related loci the mutation has occurred, a hyphen is used instead of the capital letter (e.g., *ara-23*). It is essential in papers reporting the isolation of new mutants that allele numbers be given to the mutations. For *Escherichia coli*, there is a registry of such numbers: the Coli Genetic Stock Center (http://cgsc.biology.yale.edu/). For the genus *Salmonella*, the registry is the *Salmonella* Genetic Stock Centre (http://people.ucalgary.ca/~kesander/). For the genus *Bacillus*, the registry is the *Bacillus* Genetic Stock Center (http://www.bgsc.org/).

(v) The use of superscripts with genotypes (other than + to indicate wild-type alleles) should be avoided. Designations indicating amber mutations (Am), temperature-sensitive mutations (Ts), constitutive mutations (Con), cold-sensitive mutations (Cs), production of a hybrid protein (Hyb), and other important phenotypic properties should follow the allele number [e.g., *araA230*(Am) *hisD21*(Ts)]. All other such designations of phenotype must be defined at the first occurrence. If superscripts must be used, they must be approved by the editor and defined at the first occurrence in the text.

Subscripts may be used in two situations. Subscripts may be used to distinguish between genes (having the same name) from different organisms or strains; e.g., $his_{E.\ coli}$ or $his_{K\text{-}12}$ for the *his* gene of *E. coli* or strain K-12, respectively, may be used to distinguish this gene from the *his* gene in another species or

strain. An abbreviation may also be used if it is explained. Similarly, a subscript is also used to distinguish between genetic elements that have the same name. For example, the promoters of the *gln* operon can be designated $glnAp_1$ and $glnAp_2$. This form departs slightly from that recommended by Bachmann and Low (e.g., *desC1p*).

(vi) Deletions are indicated by the symbol Δ placed before the deleted gene or region, e.g., Δ*trpA432*, Δ(*aroP-aceE*)*419*, or Δ(*hisQ-hisJo*)*1256*. Similarly, other symbols can be used (with appropriate definition). Thus, a fusion of the *ara* and *lac* operons can be shown as Φ(*ara-lac*)95. Likewise, Φ(*araB′-lacZ*$^+$)*96* indicates that the fusion results in a truncated *araB* gene fused to an intact *lacZ* gene, and Φ(*malE-lacZ*)*97*(Hyb) shows that a hybrid protein is synthesized. An inversion is shown as IN(*rrnD-rrnE*)*1*. An insertion of an *E. coli his* gene into plasmid pSC101 at zero kilobases (0 kb) is shown as pSC101 Ω(0kb::K-12*hisB*)*4*. An alternative designation of an insertion can be used in simple cases, e.g., *galT236*::Tn*5*. The number *236* refers to the locus of the insertion, and if the strain carries an additional *gal* mutation, it is listed separately. Additional examples, which utilize a slightly different format, can be found in the papers by Campbell et al. and Novick et al. cited below. It is important in reporting the construction of strains in which a mobile element was inserted and subsequently deleted that this fact be noted in the strain table. This can be done by listing the genotype of the strain used as an intermediate in a table footnote or by making a direct or parenthetical remark in the genotype, e.g., (F$^-$), ΔMu *cts*, or *mal*::ΔMu *cts*::*lac*. In setting parenthetical remarks within the genotype or dividing the genotype into constituent elements, parentheses and brackets are used without special meaning; brackets are used outside parentheses. To indicate the presence of an episome, parentheses (or brackets) are used (λ, F$^+$). Reference to an integrated episome is indicated as described above for inserted elements, and an exogenote is shown as, for example, W3110/F′8(*gal*$^+$).

For information about the symbols in current use, consult Berlyn (Microbiol Mol Biol Rev **62:**814–984, 1998) for *E. coli* K-12, Sanderson and Roth (Microbiol Rev **52:**485–532, 1988) for *Salmonella* serovar Typhimurium, Holloway et al. (Microbiol Rev **43:**73–102, 1979) for the genus *Pseudomonas*, Piggot and Hoch (Microbiol Rev **49:**158–179, 1985) for *Bacillus subtilis*, Perkins et al. (Microbiol Rev **46:**426–570, 1982) for *Neurospora crassa*, and Mortimer and Schild (Microbiol Rev **49:** 181–213, 1985) for *Saccharomyces cerevisiae*. For yeasts, *Chlamydomonas* spp., and several fungal species, symbols such as those given in the *Handbook of Microbiology*, 2nd ed. (A. I. Laskin and H. A. Lechevalier, ed., CRC Press, Inc., Cleveland, OH, 1988), should be used.

Conventions for naming genes. It is recommended that (entirely) new genes be given names that are mnemonics of their function, avoiding names that are already assigned and earlier or alternative gene names, irrespective of the bacterium for which such assignments have been made. Similarly, it is recommended that, whenever possible, orthologous genes present in different organisms receive the same name. When homology is not apparent or the function of a new gene has not been established, a provisional name may be given by one of the following methods. (i) The gene may be named on the basis of its map location in the style *yaaA*, analogous to the style used for recording transposon insertions (*zef*) as discussed below. A list of such names in use for *E. coli* has been published by Rudd (Microbiol Mol Biol Rev **62:**985–1019, 1998). (ii) A provisional name may be given in the style described by Demerec et al. (e.g., *usg*, gene upstream of *folC*). Such names should be unique, and names such as *orf* or *genX* should not be used. For reference, the *E. coli* Genetic Stock Center's database includes an updated listing of *E. coli* gene names and gene products. It is accessible on the Internet (http://cgsc.biology.yale.edu/index.php). A list can also be found in the work of Riley (Microbiol Rev **57:**862–952, 1993). For the genes of other bacteria, consult the references given above.

For prokaryotes, gene names should not begin with prefixes indicating the genus and species from which the gene is derived. (However, subscripts may be used where necessary to distinguish between genes from different organisms or strains, as described in section v of "Bacteria" above.) For eukaryotes, such prefixes may be used for clarity when discussing genes with the same name from two different organisms (e.g., Sc*URA3* versus Ca*URA3*); the prefixes are not considered part of the gene name proper and are not italicized.

Locus tags. Locus tags are systematic, unique identifiers that are assigned to each gene in GenBank. All genes mentioned in a manuscript should be traceable to their sequences by the reader, and locus tags may be used for this purpose in manuscripts to identify uncharacterized genes. In addition, authors should check GenBank to make sure that they are using the correct, up-to-date format for locus tags (e.g., uppercase versus lowercase letters and the presence or absence of an underscore, etc.). Locus tag formats vary between different organisms and also may be updated for a given organism, so it is important to check GenBank at the time of manuscript preparation.

"Mutant" versus "mutation." Keep in mind the distinction between a mutation (an alteration of the primary sequence of the genetic material) and a mutant (a strain carrying one or more mutations). One may speak about the mapping of a mutation, but one cannot map a mutant. Likewise, a mutant has no genetic locus, only a phenotype.

"Homology" versus "similarity." For use of terms that describe relationships between genes, consult the articles by Theissen (Nature **415:**741, 2002) and Fitch (Trends Genet **16:** 227–231, 2000). "Homology" implies a relationship between genes that have a common evolutionary origin; partial homology is not recognized. When sequence comparisons are discussed, it is more appropriate to use the term "percent sequence similarity" or "percent sequence identity," as appropriate. When using "percent sequence similarity," the method/algorithm used to calculate the percentage should be stated.

Strain designations. Do not use a genotype as a name (e.g., "subsequent use of *leuC6* for transduction"). If a strain designation has not been chosen, select an appropriate word combination (e.g., "another strain containing the *leuC6* mutation").

"Natural" versus "artificial" transformation. Natural transformation is a process whereby the recipient cell has the inherent capacity to take up and integrate exogenous DNA into its

genome. As such, natural transformation is part of the biology of the recipient cell line and should not be confused with processes through which integration of DNA is forced upon recipient cells.

Viruses. The genetic nomenclature for viruses differs from that for bacteria. In most instances, viruses have no phenotype, since they have no metabolism outside host cells. Therefore, distinctions between phenotype and genotype cannot be made. Superscripts are used to indicate hybrid genomes. Genetic symbols may be one, two, or three letters. For example, a mutant strain of λ might be designated λ *Aam11 int2 red114 cI857*; this strain carries mutations in genes *cI*, *int*, and *red* and an amber-suppressible (Am) mutation in gene *A*. A strain designated λ att^{434} imm^{21} would represent a hybrid of phage λ that carries the immunity region (*imm*) of phage 21 and the attachment (*att*) region of phage 434. Host DNA insertions into viruses should be delineated by square brackets, and the genetic symbols and designations for such inserted DNA should conform to those used for the host genome. Genetic symbols for phage λ can be found in reports by Szybalski and Szybalski (Gene **7**:217–270, 1979) and Echols and Murialdo (Microbiol Rev **42**:577–591, 1978).

Eukaryotes. FlyBase (http://flybase.org/) is the genetic nomenclature authority for *Drosophila melanogaster*. WormBase (http://www.wormbase.org/#01-23-6) is the genetic nomenclature authority for *Caenorhabditis elegans*. When naming genes for *Aspergillus* species, the nomenclature guidelines posted at http://www.aspergillusgenome.org/Nomenclature.shtml should be followed, and the *Aspergillus* Genome Database (http://www.aspgd.org/) should be searched to ensure that any new name is not already in use. The *Saccharomyces* Genome Database (http://www.yeastgenome.org/) and the *Candida* Genome Database (http://www.candidagenome.org/) are authorities for *Saccharomyces cerevisiae* and *Candida albicans* genetic nomenclature, respectively.

For more information about the genetic nomenclature of eukaryotes, see the Instructions to Authors for *Molecular and Cellular Biology*.

Transposable elements, plasmids, and restriction enzymes. Nomenclature of transposable elements (insertion sequences, transposons, and phage Mu, etc.) should follow the recommendations of Campbell et al. (Gene **5**:197–206, 1979), with the modifications given in section vi of "Bacteria" above. The Internet site where insertion sequences of eubacteria and archaea are described and new sequences can be recorded is https://www-is.biotoul.fr.

The system of designating transposon insertions at sites where there are no known loci, e.g., *zef-123*::Tn*5*, has been described by Chumley et al. (Genetics **91**:639–655, 1979). The nomenclature recommendations of Novick et al. (Bacteriol Rev **40**:168–189, 1976) for plasmids and plasmid-specified activities, of Low (Bacteriol Rev **36**:587–607, 1972) for F′ factors, and of Roberts et al. (Nucleic Acids Res **31**:1805–1812, 2003) for restriction enzymes, DNA methyltransferases, homing endonucleases, and their genes should be used when possible. The nomenclature for recombinant DNA molecules constructed *in vitro* follows the nomenclature for insertions in general. DNA inserted into recombinant DNA molecules should be described by using the gene symbols and conventions for the organism from which the DNA was obtained.

Tetracycline resistance determinants. The nomenclature for tetracycline resistance determinants is based on the proposal of Levy et al. (Antimicrob Agents Chemother **43**:1523–1524, 1999). The style for such determinants is, e.g., Tet B; the space helps distinguish the determinant designation from that for phenotypes and proteins (TetB). The above-referenced article shows the correct format for genes, proteins, and determinants in this family.

ABBREVIATIONS AND CONVENTIONS

Verb Tense

ASM strongly recommends that for clarity you use the **past** tense to narrate particular events in the past, including the procedures, observations, and data of the study that you are reporting. Use the present tense for your own general conclusions, the conclusions of previous researchers, and generally accepted facts. Thus, most of the abstract, Materials and Methods, and Results will be in the past tense, and most of the introduction and some of the Discussion will be in the present tense.

Be aware that it may be necessary to vary the tense in a single sentence. For example, it is correct to say "White (30) demonstrat*ed* that XYZ cells *grow* at pH 6.8," "Figure 2 shows that ABC cells failed to grow at room temperature," and "Air *was* removed from the chamber and the mice *died*, which *proves* that mice *require* air." In reporting statistics and calculations, it is correct to say "The values for the ABC cells *are* statistically significant, indicating that the drug inhibit*ed* . . . ."

For an in-depth discussion of tense in scientific writing, see *How To Write and Publish a Scientific Paper*, 7th ed.

Abbreviations

General. Abbreviations should be used as an aid to the reader rather than as a convenience to the author, and therefore their **use should be limited**. Abbreviations other than those recommended by the IUPAC-IUB (*Biochemical Nomenclature and Related Documents*, 1992) should be used only when a case can be made for necessity, such as in tables and figures.

It is often possible to use pronouns or to paraphrase a long word after its first use (e.g., "the drug" or "the substrate"). Standard chemical symbols and trivial names or their symbols (folate, Ala, and Leu, etc.) may also be used.

Define each abbreviation and introduce it in parentheses the first time it is used; e.g., "cultures were grown in Eagle minimal essential medium (MEM)." Generally, eliminate abbreviations that are not used at least three times in the text (including tables and figure legends).

Not requiring introduction. In addition to abbreviations for Système International d'Unités (SI) units of measurement, other common units (e.g., bp, kb, and Da), and chemical symbols for the elements, the following should be used without definition in the title, abstract, text, figure legends, and tables:

DNA (deoxyribonucleic acid)	cRNA (complementary RNA)
cDNA (complementary DNA)	RNase (ribonuclease)
RNA (ribonucleic acid)	DNase (deoxyribonuclease)

rRNA (ribosomal RNA)
mRNA (messenger RNA)
tRNA (transfer RNA)
AMP, ADP, ATP, dAMP, ddATP, and GTP, etc. (for the respective 5′ phosphates of adenosine and other nucleosides) (add 2′-, 3′-, or 5′- when needed for contrast)
ATPase and dGTPase, etc. (adenosine triphosphatase and deoxyguanosine triphosphatase, etc.)
NAD (nicotinamide adenine dinucleotide)
NAD^+ (nicotinamide adenine dinucleotide, oxidized)
NADH (nicotinamide adenine dinucleotide, reduced)
NADP (nicotinamide adenine dinucleotide phosphate)
NADPH (nicotinamide adenine dinucleotide phosphate, reduced)
$NADP^+$ (nicotinamide adenine dinucleotide phosphate, oxidized)
poly(A) and poly(dT), etc. (polyadenylic acid and polydeoxythymidylic acid, etc.)
oligo(dT), etc. (oligodeoxythymidylic acid, etc.)
UV (ultraviolet)
PFU (plaque-forming units)
CFU (colony-forming units)
MIC (minimal inhibitory concentration)
Tris [tris(hydroxymethyl) aminomethane]
DEAE (diethylaminoethyl)
EDTA (ethylenediamine-tetraacetic acid)
EGTA (ethylene glycol-bis[β-aminoethyl ether]-*N,N,N′,N′*-tetraacetic acid)
HEPES (*N*-2-hydroxyethyl-piperazine-*N′*-2-ethanesulfonic acid)
PCR (polymerase chain reaction)
AIDS (acquired immunodeficiency syndrome)

Abbreviations for cell lines (e.g., HeLa) also need not be defined.

The following abbreviations should be used without definition in tables:

amt (amount)
approx (approximately)
avg (average)
concn (concentration)
diam (diameter)
expt (experiment)
exptl (experimental)
ht (height)
mo (month)
mol wt (molecular weight)
no. (number)
prepn (preparation)
SD (standard deviation)
SE (standard error)
SEM (standard error of the mean)
sp act (specific activity)
sp gr (specific gravity)
temp (temperature)
vol (volume)
vs (versus)
wk (week)
wt (weight)
yr (year)

Reporting Numerical Data

Standard metric units are used for reporting length, weight, and volume. For these units and for molarity, use the prefixes m, μ, n, and p for 10^{-3}, 10^{-6}, 10^{-9}, and 10^{-12}, respectively. Likewise, use the prefix k for 10^3. Avoid compound prefixes such as mμ or μμ. Parts per million (ppm) may be used when that is the common measure for the science in that field. Units of temperature are presented as follows: 37°C or 324 K.

When fractions are used to express such units as enzymatic activities, it is preferable to use whole units, such as g or min, in the denominator instead of fractional or multiple units, such as μg or 10 min. For example, "pmol/min" is preferable to "nmol/10 min," and "μmol/g" is preferable to "nmol/μg." It is also preferable that an unambiguous form, such as exponential notation, be used; for example, "μmol g^{-1} min^{-1}" is preferable to "μmol/g/min." Always report numerical data in the applicable SI units.

Representation of data as accurate to more than two significant figures must be justified by presentation of appropriate statistical analyses.

For a review of some common errors associated with statistical analyses and reports, plus guidelines on how to avoid them, see the articles by Olsen (Infect Immun **71**:6689–6692, 2003; Infect Immun **82**:916–920, 2014).

For a review of basic statistical considerations for virology experiments, see the article by Richardson and Overbaugh (J Virol **79**:669–676, 2005).

Statistics

If biological variation within a treatment (coefficient of variation, the standard deviation divided by the mean) is small (less than 10%) and the difference among treatment means is large (greater than 3 standard deviations), it is not necessary to report statistics. If the data do not meet these criteria, however, the authors must include an appropriate statistical analysis (e.g., Student's *t* test, analysis of variance, or Tukey's test, etc.). Statistics should represent the variation among biological units (e.g., replicate incubations) and not just the variation due to method of analysis.

Phylogenetic trees based on nucleotide or amino acid sequence alignments must be supported by appropriate statistical analyses of tree stability (e.g., bootstrap analysis), and nonsupported branches (e.g., bootstrap coefficients below 50%) should be collapsed. A copy of the alignment should be available for examination by the editor or the reviewers upon request.

For a review of some common errors associated with statistical analyses and reports, plus guidelines on how to avoid them, see the articles by Olsen (Infect Immun **71**:6689–6692, 2003; Infect Immun **82**:916–920, 2014).

For a review of basic statistical considerations for virology experiments, see the article by Richardson and Overbaugh (J Virol **79**:669–676, 2005).

Equations

In mathematical equations, indicate the order of operations clearly by enclosing operations in parentheses, brackets, and braces, in that order: $(a + b) \times c$ or $a + (b \times c)$, $100 \times \{[(a/b) \times c] + d\}$ or $100 \times [a/][(b \times c) + d]$. Italicize variables and constants (but not numerals), and use roman type for designations: E_o, E_h, M_r, K_m, K_s, $a + 2b = 1.2$ mM, Ca^{2+} $V_{max} = \exp(1.5x + y)$, $BOD = 2.7x^2$.

Isotopically Labeled Compounds

For simple molecules, isotopic labeling is indicated in the chemical formula (e.g., $^{14}CO_2$, $^3H_2$, and $H_2{}^{35}SO_4$). Brackets are not used when the isotopic symbol is attached to the name of a compound that in its natural state does not contain the element (e.g., ^{32}S-ATP) or to a word that is not a specific chemical name (e.g., ^{131}I-labeled protein, ^{14}C-amino acids, and ^{3}H-ligands).

For specific chemicals, the symbol for the isotope introduced is placed in brackets directly preceding the part of the name that describes the labeled entity. Note that configuration symbols and modifiers precede the isotopic symbol. The following examples illustrate correct usage.

[^{14}C]urea
L-[*methyl*-^{14}C]methionine
[2,3-^{3}H]serine
[α-^{14}C]lysine
[γ-^{32}P]ATP
UDP-[U-^{14}C]glucose
E. coli [^{32}P]DNA
fructose 1,6-[1-^{32}P]bisphosphate