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SELEÇÃO DE BACTÉRIAS LÁCTICAS COM POTENCIAL PROBIÓTICO PROVENIENTES DE QUEIJO COLONIAL

FRANCISCO BELTRÃO – PR

(JULHO/2020)

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DISSERTAÇÃO apresentada ao Programa de Pós-graduação *Stricto Sensu* em Ciências Aplicadas à Saúde, nível Mestrado, do Centro de Ciências da Saúde, da Universidade Estadual do Oeste do Paraná, como requisito parcial para obtenção do título de Mestre em Ciências Aplicadas à Saúde.

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JULIANA ALEXSANDRA MACHADO ANDRÉ

SELEÇÃO DE BACTÉRIAS LÁCTICAS COM POTENCIAL PROBIÓTICO PROVENIENTES DE QUEIJO COLONIAL

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LISTA DE ABREVIATURAS E SIGLAS

ANVISA – Agência Nacional de Vigilância Sanitária

BAL – Bactérias ácido láticas

BHI – Brain Heart Infusion

°C – Graus Celsius

CHO – Carboidratos

CO_2 – Dióxido de carbono

DCNT – Doenças Crônicas Não Transmissíveis

DM – Diabetes *mellitus*

FOSHU – *Foods for Specified Health Use*

g – Gramas

HAS – Hipertensão Arterial Sistêmica

MALDI-TOF – *Matrix Associated Laser Desorption-Ionization - Time of Flight*

MH – Müller-Hinton

mL – Mililitros

NaCl – Cloreto de sódio

PCR – Reação em Cadeia da Polimerase

pH – Potencial hidrogeniônico

RDC – Resolução de Diretoria Colegiada

µL – Microlitros

Seleção de bactérias lácticas com potencial probiótico provenientes de queijo colonial

Resumo

Os queijos artesanais, também conhecidos como queijos coloniais, são comumente produzidos na região Sudoeste do Paraná e suas técnicas de produção são transmitidas verbalmente e passadas de geração em geração, bem como apresentam uma diversificada população microbiana, tanto de bactérias desejáveis quanto indesejáveis, as quais podem deteriorar o produto e causar danos à saúde do consumidor. Dentre os microrganismos desejáveis estão as bactérias ácido lácticas, as quais apresentam-se úteis nos processos de fermentação e de preservação dos queijos, além de possuírem potencial probiótico, visto que conseguem resistir ao pH ácido do estômago, suco gástrico, sais biliares e enzimas digestivas, agindo beneficamente no intestino. Entre os diversos benefícios que as bactérias ácido lácticas proporcionam para a saúde humana, estão as ações antimicrobiana, antioxidante e anticarcinogênica, bem como, reduzem a ocorrência de doenças intestinais e intolerância à lactose. Diante desse contexto, o objetivo do presente estudo foi isolar e identificar, por meio de análises fenotípicas, bactérias ácido láticas com potencial probiótico a partir de queijos coloniais. Para tanto, amostras de queijo colonial (n=10) foram utilizadas para o isolamento de culturas bacterianas. As bactérias foram caracterizadas fenotipicamente e testadas quanto a resistência à diferentes temperaturas, capacidade de fermentação de carboidratos e capacidade de crescimento em diferentes concentrações de NaCl. Posteriormente, foram selecionados 20 isolados para análise de atividade e de suscetibilidade antimicrobiana, tolerância e resistência a meios ácidos. Observou-se que a maioria das bactérias apresentou formato de bacilos Gram-positivos e catalase negativas, todas apresentaram crescimento nas temperaturas avaliadas (10°C e 45°C) e a maioria fermentou todos os carboidratos (glicose, lactose, sorbitol e manitol) com produção de gases, caracterizando-se como heterofermentativas. Quanto à resistência a diferentes antimicrobianos, 75% demostraram-se resistentes a dois ou mais antimicrobianos. Os isolados também se apresentaram pouco sensíveis ao meio ácido, com maior tempo de sobrevivência quando o meio ácido

foi associado ao leite e ótima resistência em condições intestinais. A identificação dos microrganismos por MALDI-TOF identificou cinco isolados como sendo *Lactobacillus brevis*, dois como *Enterococcus faecium*, três como *Pediococcus acidilactici* e um como *Lactobacillus rhamnosus*. Diante de todo esse contexto, é possível inferir que as BAL presentes nos queijos coloniais analisadas apresentam potencial probiótico, merecendo destaque em pesquisas futuras, visto que possuem aspectos positivos com relação aos quesitos avaliados.

Palavras-chave: Microrganismos, caracterização fenotípica, microbiota, alimentos fermentados, antagonismo.

Selection of lactic acid bacteria with probiotic potential from colonial cheese

Abstract

Artisanal cheeses, also known as colonial cheeses, are commonly produced in the Southwest region of Paraná and their production techniques are transmitted verbally and passed down from generation to generation, as well as presenting a diverse microbial population, both desirable and undesirable bacteria, which can deteriorate the product and cause damage to the health of the consumer. Among the desirable microorganisms are the lactic acid bacteria, which are useful in the fermentation and cheeses preservation processes, in addition to having probiotic potential, since they can resist the acid pH of the stomach, gastric juice, bile salts and digestive enzymes, acting beneficially in the intestine. Among the many benefits that lactic acid bacteria provide for human health are antimicrobial, antioxidant and anticarcinogenic actions, as well as reducing the occurrence of intestinal diseases and lactose intolerance. In this context, the aim of the present study was to isolate and identify, through phenotypic analyses, lactic acid bacteria with probiotic potential from colonial cheeses. For that, samples of colonial cheese samples (n = 10) were used to the isolate of bacterial cultures. The bacteria were phenotypically characterized and tested for resistance to different temperatures, carbohydrate fermentation capacity and growth capacity at different NaCl concentrations. Subsequently, 20 isolates were selected for analysis of antimicrobial activity and susceptibility, tolerance, and resistance to acidic media. It was observed that most bacteria presented Gram-positive bacilli and negative catalase, all showed growth at the temperatures evaluated (10°C and 45°C) and most fermented all carbohydrates (glucose, lactose, sorbitol and mannitol) with gas production, characterized as heterofermentative. Regarding resistance to different antimicrobials, 75% were resistant to two or more antimicrobials. The isolates were also showed little sensitivity to the acidic medium, with longer survival time when the acidic medium was associated with milk and excellent resistance under intestinal conditions. The identification of microorganisms by MALDI-TOF identified five isolates as *Lactobacillus brevis*, two as *Enterococcus faecium*, three as *Pediococcus*

acidilactici and one as *Lactobacillus rhamnosus*. Considering this context, it is possible to infer that the BAL present in the colonial cheeses analyzed have probiotic potential, deserving prominence in future research, since they have positive aspects in relation to the items evaluated.

Keywords: Microorganisms, phenotypic characterization, microbiota, fermented foods, antagonism.

SUMÁRIO

1. INTRODUÇÃO GERAL

Os queijos artesanais, popularmente denominados como queijos coloniais, são comumente produzidos na região Sudoeste do Paraná e suas técnicas de produção são transmitidas verbalmente e passadas de geração em geração. Devido às técnicas de manejo e por serem produzidos, na maioria dos casos, com leite cru e sem adição de um inóculo inicial, os queijos coloniais apresentam uma diversificada população microbiana indesejada, a qual apresenta-se como um fator de deterioração do produto e perigo microbiológico para os consumidores (HERMANNS, 2013; PEHRSON, 2017).

Além desses microrganismos indesejáveis, estão presentes naturalmente nos queijos coloniais as bactérias ácido láticas (BAL), essenciais para o processo de fermentação e uma das formas mais antigas de preservação. Isso ocorre devido à redução do pH e consequente produção de ácidos orgânicos, como o ácido lático, a partir da fermentação de carboidratos (CHO) disponíveis, tornando-se o principal efeito antagonista contra diferentes microrganismos (NASCIMENTO, 2007; CORBO *et al.,* 2009; HERMANNS, 2013).

Devido ao potencial probiótico, as BAL têm sido incorporadas aos queijos, por apresentarem características adequadas, como sua capacidade tamponante e seu elevado teor de gordura, oferecendo proteção às bactérias durante a passagem pelo trato gastrointestinal (BACK *et al.,* 2013). Após ingeridas, as bactérias probióticas devem ser capazes de sobreviver às condições adversas presentes no trato gastrointestinal, como suco gástrico, sais biliares e enzimas digestivas, bem como devem manter sua viabilidade e atividade metabólica no intestino, para então atuar beneficamente sobre as funções do organismo humano (SAAD, 2006; ARAÚJO, 2007).

Dentre os benefícios gerados pelo efeito probiótico das BAL pode-se destacar a atividade antimicrobiana, a atividade antioxidante, o controle das infecções intestinais, a melhora na absorção de alguns nutrientes, a melhora na utilização de lactose e no alívio dos sintomas de intolerância a esse açúcar, a redução dos níveis de colesterol, o efeito anticarcinogênico e melhora da resposta imune em decorrência da produção de anticorpos (SAARELA *et al.,* 2002;

VASILJEVIC; SHAH, 2008; SCHMID *et al.,* 2006; BACK *et al.,* 2013).

Diante desse contexto, destaca-se a importância do desenvolvimento de estudos que sejam capazes de caracterizar a colonização bacteriana dos queijos coloniais, em especial as BAL, a fim de incentivar o consumo de produtos regionais, bem como favorecer a economia local.

1.1 Alimentos Funcionais

A expectativa de vida das pessoas vem aumentando com o passar dos anos e ao mesmo tempo tem crescido a incidência das doenças crônicas, tais como Diabetes *mellitus* (DM), Hipertensão Arterial Sistêmica (HAS) e câncer. Nesse sentido, a população vem adotando hábitos alimentares mais saudáveis, buscando um equilíbrio alimentar. Foi a busca por essa alimentação equilibrada que despertou o interesse por alguns alimentos que, além de suprir as necessidades básicas do organismo, também previnem algumas doenças (CARDOSO, 2012).

Segundo Vidal *et al*. (2012), alimentos funcionais são aqueles que produzem efeitos fisiológicos ou metabólicos por meio do desempenho de algum nutriente, na manutenção das funções do organismo humano, ou seja, eles proporcionam um benefício fisiológico adicional, além daquele de satisfazer às necessidades nutricionais básicas.

Um alimento pode ser considerado funcional se for demonstrado que pode afetar beneficamente uma ou mais funções alvo no organismo, além de possuir os adequados efeitos nutricionais, de maneira que seja tanto relevante para o bem-estar e a saúde, quanto para a redução do risco de doenças (SILVA, ORLANDELLI, 2019). Os alimentos funcionais são alimentos capazes de combinar produtos comestíveis de alta flexibilidade com moléculas biologicamente ativas, como estratégia para corrigir distúrbios metabólicos, resultando em redução dos riscos de doenças e manutenção da saúde (LAMOUNIER, 2015).

Lima *et al*. (2017) destacam alguns critérios para determinação de um alimento funcional, tais como: exercer ação metabólica ou fisiológica que contribua para a saúde física e para a diminuição de morbidades crônicas; integrar a alimentação usual; os efeitos positivos devem ser obtidos em quantidades não tóxicas, perdurando mesmo após suspensão de sua ingestão; e, por fim, não são destinados ao tratamento ou cura das doenças, apenas previnem seu

acontecimento e caso já estejam instaladas ajudam o organismo a combatê-las de forma mais eficaz.

Os alimentos funcionais podem ser encontrados para consumo humano nas formas artificiais e naturais. Os alimentos funcionais artificiais são fabricados por empresas especializadas e autorizadas. Já as formas naturais (ou compostos bioativos)[1] são os alimentos que contêm ácidos graxos (linoléico, ômega-3 e 6, e limonoides), fibras, compostos fenólicos (resveratrol, isoflavona e zeaxantina); carotenoides (betacaroteno, licopeno, luteína); fitoquímicos, peptídeos ativos (arginina e glutamina), prebióticos (inulina e oligofrutose ou frutooligossacarídeo) e os probióticos como *Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus lactis* e *Lactobacillus bulgaricus* (MOREIRA *et al*., 2017).

Há um aumento no comércio e no consumo dos alimentos funcionais, que além de apresentarem características nutricionais e tecnológicas peculiares, atendem às exigências do consumidor que busca alimentos inovadores (ORTEGA, *et al*, 2016). A indústria de laticínios vem se destacando nesse aspecto com o maior número de produtos funcionais, através da adição de probióticos e prebióticos em alimentos como o iogurte e os leites fermentados no geral (HUANG; ZHOU, 2017).

1.2 Microbiota do trato gastrointestinal

As bactérias coabitam normalmente com humanos e se encontram associados a vários tecidos do corpo humano, incluindo todo o trato gastrointestinal. As que comumente habitam a cavidade oral são os estreptococos, porém não colonizam o estômago em grandes quantidades, devido ao baixo pH e ao trânsito rápido desse órgão, sendo os principais exemplos os lactobacilos, enterococos, helicobactérias e bacilos (PAIXÃO, CASTRO, 2016).

O duodeno também tende a ser ácido e apresenta trânsito rápido. Além disso, recebe secreções pancreáticas e biliares, que criam um ambiente hostil para os microrganismos, de modo que, ali predominam os lactobacilos e estreptococos. No

[1] Compostos bioativos são substâncias que compõe a matriz do alimento e que melhoram o bem-estar geral e a saúde. Os componentes ativos ligados a estes benefícios incluem prebióticos, fibras, probióticos, peptídeos, proteínas, vitaminas, minerais e ácidos graxos ômega-3. As substâncias bioativas compreendem, entre outras, os carotenóides, os fitoesteróis, os flavonóides, os fosfolipídeos, os organossulfurados e os polifenóis.

jejuno e, particularmente, no íleo, há aumento gradual no número e na diversidade das bactérias presentes (SANDERS, 2011), e o colón contém a maior parte dos microrganismos gastrointestinais. É importante destacar que antes do nascimento, não existem microrganismos presentes no trato gastrointestinal, mas a colonização ocorre rapidamente durante ou após o parto (GRITZ; BHANDARI, 2015).

Os microrganismos intestinais podem ser comensais (microrganismos colonizadores nativos de um indivíduo) ou temporários (microrganismos de passagem). Além disso, esses microrganismos podem ser benéficos, potencialmente nocivos ou patogênicos. Os microrganismos considerados benéficos geralmente fermentam carboidratos, não produzem toxinas e podem proporcionar uma série de potenciais benefícios para o hospedeiro, como a interação com o sistema imunológico e a inibição competitiva de patógenos. Esses microrganismos incluem os gêneros *Bifidobacterium*, *Eubacterium* e *Lactobacillus* (KAPEL *et al.,* 2014).

O intestino delgado é o principal alvo de muitas infecções exógenas, como as causadas por rotavírus, *Salmonella* Typhimurium e algumas cepas de *Escherichia coli*, geralmente contraídos a partir de água ou alimentos contaminados. No entanto, todos os indivíduos carregam microrganismos com potencial patogênico oportunista (SANDERS, 2011).

O trato gastrointestinal é descrito como o maior órgão imunológico do corpo humano. Representa a maior área de contato da mucosa do hospedeiro com o ambiente e, contem até 80% de todas as células que produzem anticorpos. A microbiota intestinal também é uma parte essencial do sistema de defesa do corpo humano. A integridade do revestimento epitelial do trato gastrointestinal é essencial para a saúde e o rompimento dessa barreira intestinal pode aumentar o risco de certos distúrbios ou doenças intestinais (GIBSON *et al*., 2011).

1.3 Bactérias ácido láticas

As BAL são microrganismos sob a forma de cocos ou bacilos Gram-positivos, catalase-negativas, não formadoras de esporos filogeneticamente distintas, imóveis, anaeróbicas facultativas, capazes de realizar a fermentação em anaerobiose, bem como em aerobiose, mas de uma forma mais lenta. Produzem o ácido láctico, como o principal produto final da fermentação dos açúcares (CABRAL,

et al, 2016). Estão amplamente distribuídas na natureza e podem ser encontradas em diferentes produtos alimentares como fermentados, carnes, derivados lácteos, vegetais e bebidas (CARVALHO, *et al*, 2017).

As BAL têm sido amplamente utilizadas na produção de iogurtes, queijos, manteiga, bebidas, leites fermentados, produtos cárneos, entre outros produtos, conferindo aos produtos características sensoriais únicas como aroma, textura e *flavor* (BRUNO; CARVALHO, 2009; WANG; CUI; QU, 2018).

Além das diversas substâncias com características sensoriais, as BAL também produzem substâncias antimicrobianas capazes de interferir no metabolismo e na multiplicação de bactérias patogênicas, tais como, ácidos orgânicos, peróxido de hidrogênio, dióxido de carbono, diacetil e bacteriocinas, que tornam o meio desfavorável para a multiplicação de patógenos e algumas bactérias deteriorantes e atuam favoravelmente nos produtos alimentares, fazendo parte dos microrganismos capazes de exercer efeitos benéficos ao hospedeiro (O'SULLIVAN *et al*., 2015).

As BAL compreendem 13 gêneros: *Carnobacterium*, *Enterococcus*, *Lactococcus*, *Lactobacillus*, *Lactosphaera*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Paralactobacillus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* e *Weissella*, sendo considerados ainda, os gêneros: *Aerococcus*, *Alloiococcus*, *Dolosigranulum*, *Globicatella* e *Melissococcus* (SILVA, 2016). Contudo, os gêneros *Lactobacillus* e *Enterococcus,* são mais comumente utilizados como probióticos (MENDES *et al*., 2014).

O gênero *Lactobacillus* é definido como células alongadas, em forma de bastonete, não produtores de esporos, termófilos, microaerófilos, Gram-positivo e produtoras de ácidos, em grande parte lático, a partir de carboidratos (VANDENPLAS; HUYS; DAUBE, 2015). Microscopicamente, essas bactérias são imóveis e apresentam hastes finas que variam em comprimento de longo para curto. Também podem aparecer como coneiformes, uma morfologia dobrada ou tendem a crescer em cadeias. A maioria das espécies de lactobacilos é anaeróbia facultativa (OLIVEIRA, 2018).

O gênero *Lactobacillus* reúne cerca de 140 espécies que podem ser encontradas no leite cru, em produtos fermentados, na carne e em frutas (SILVA, 2016). Desempenha importante papel na produção e na preservação dos alimentos e é componente de vários tipos de fermentos, como, por exemplo, na produção de

leites fermentados e na maturação de diversos tipos de queijos. Também contribui para a manutenção do equilíbrio da microbiota intestinal, constituindo componentes importantes na produção de probióticos para a alimentação humana e animal (CARVALHO, 2017). Muitas cepas de *Lactobacillus* foram caracterizadas como probióticos e foram notificadas para exercer benefícios para saúde do consumidor (YERLIKAYA, 2019).

Embora as BAL englobem diversos gêneros, são agrupadas de acordo com o produto final da sua fermentação, sendo divididas em: homofermentativas, ou seja, que produzem ácido láctico como principal produto da fermentação da glicose e heterofermentativas, que além do ácido láctico, formam outras substâncias, como dióxido de carbono, ácido acético e etanol (SHORI, 2015).

A identificação das BAL pode ser realizada mediante a multiplicação em meios de cultura seletivos, com posterior identificação do gênero e/ou espécie utilizando técnicas bioquímicas, fisiológicas e moleculares ou exclusivamente utilizando métodos moleculares, como a Reação em Cadeia da Polimerase - PCR (RESENDE, 2011).

O método clássico de identificação das BAL é a microbiologia tradicional, na qual é necessário o isolamento bacteriano que é realizado por meio do cultivo microbiológico. Para isso, há uma série de meios de cultura disponíveis; porém, os mais utilizados são o ágar M17 e o ágar Rogosa acidificado ou o ágar MRS, desenvolvido por Man, Rogosa e Sharpe, para isolamento de bactérias com morfologia de cocos e bacilos (MENDES *et al*., 2015).

As colônias isoladas são purificadas, geralmente em ágar MRS e submetidas aos seguintes testes: coloração diferencial de Gram, verificação da morfologia, teste de atividade de catalase e de produção de ácido (MOTA *et al.,* 2015). Os microrganismos Gram-positivos, catalase negativo, produtores de ácido, com morfologia de cocos ou bacilos são considerados BAL. Como critério funcional, cepas de BAL probióticas devem sobreviver à passagem ao trato gastrointestinal e, portanto, serem capazes de tolerar as condições ácidas do estômago, resistir às enzimas digestivas e aos ácidos biliares no início do intestino delgado, aderir à superfície da mucosa intestinal e, assim, assegurar benefícios clinicamente validados para a saúde dos consumidores (CABRAL, 2016).

A resistência à simulação dos sucos gástrico e intestinal estão entre os ensaios *in vitro* que são frequentemente sugeridos para a avaliação da cepa com

potencial probiótico (BARBOSA; GIBBIS; TEIXEIRA; 2010).

As culturas de BAL utilizadas em alimentos não possuem potencial patogênico, além de serem excelentes produtoras de substâncias antimicrobianas, criando um microambiente desfavorável a diversos microrganismos, inclusive aqueles com potencial patogênico, sendo esta característica a base de inúmeros métodos de conservação de alimentos por fermentação. As condições ácidas do meio melhoram a competitividade das BAL, que apresentam maior tolerância ao baixo pH extra e intracelular, comparado às demais bactérias (PATEL; GOYAL, 2012).

A grande variedade e o número de aplicações de BAL aumentam a necessidade de correlacionar características industriais e clinicamente importantes com informações genômicas para examinar as possibilidades de exploração de seu potencial metabólico, melhorando assim seu uso em aplicações biotecnológicas e relacionadas à saúde animal e humana (ORTEGA *et al*., 2016).

O estudo do potencial probiótico e biotecnológico de culturas de BAL é interessante à indústria nutracêutica e alimentícia, pois promovem várias informações sobre suas características que são avaliadas para realizar em seus produtos a utilização adequada desses microrganismos, garantindo assim a segurança dos produtos funcionais para novas terapias (LIMA *et al*., 2017).

1.4 Probióticos

Os probióticos são microrganismos vivos que quando administrados de forma adequada, conferem benefícios à saúde do hospedeiro, podendo ser incluídos na preparação de uma ampla gama de produtos, como alimentos, medicamentos e suplementos dietéticos. As espécies de *Lactobacillus* e *Enterecoccus* são as mais comumente usadas como probióticos (BRÜSSOW, 2019).

Para que os microrganismos possam ser considerados probióticos devem resistir à passagem pelo estômago e intestino delgado para seguirem até o intestino grosso onde possam promover seus benefícios. Para que isso aconteça devem resistir ao suco gástrico e sais biliares, aderirem ao muco ou epitélio intestinal e ter viabilidade até o consumo final, além de comprovação *in vivo* e *in vitro* das doses de ingestão recomendadas (ZACARCHENCO *et al*., 2013; SANDERS *et al*., 2019).

Os probióticos são capazes de fermentar os prebióticos, favorecendo uma

vantagem de competição, melhorando sua sobrevivência no trato gastrointestinais, pois a fermentação é uma fonte de energia (SCHNEIDER, 2016). Um produto contendo prebióticos e probióticos combinados é considerado simbiótico, onde no qual o conjunto desses dois substratos favorece-os mutuamente e o consumo confere inúmeros benefícios ao indivíduo (PAIXÃO; CASTRO, 2016).

Probióticos são capazes de modular algumas características fisiológicas do trato gastrointestinais, como a imunidade da mucosa e a permeabilidade intestinal (SCHNEIDER, 2016). Vários fatores externos podem interferir na microbiota normal do nosso intestino, como a dieta, o uso de antibióticos, estresse, fumo, tratamentos quimioterápicos e radioterapia, além do envelhecimento (CABRAL *et al.*, 2016).

Os produtos probióticos são desenvolvidos para reduzir as doenças fisiológicas em diferentes áreas do corpo. Apesar de o trato gastrointestinais ser o alvo mais importante da maioria dos probióticos, outras áreas do corpo, como a cavidade oral, o trato urogenital e a pele também são consideradas. Os probióticos desempenham importantes papéis na medicina e, também, na odontologia (PINO, *et al*., 2019).

Os principais benefícios do uso de probióticos são:

> Auxiliar no tratamento de desordens intestinais como diarreia aguda, síndrome do intestino irritável, doença de Chron, constipação e colite pseudomembranosa; prevenir infecções do trato reprodutivo e urinário; induzir resposta imune que tenham efeito sistêmico, como por exemplo para o controle de inflamações na pele; prevenir infecções do trato respiratório; redução da colonização de patógenos; síntese de vitaminas; aprimoramento do trânsito gastrointestinal; alívio da intolerância à lactose; efeitos imunomoduladores; regulação da pressão arterial; redução dos níveis séricos do colesterol; redução da microbiota que causa a cárie; redução dos níveis de *Candida* sp. na saliva de idosos (SOUZA *et al*., 2011; VANDENPLAS *et al*., 2014; MOKOENA, 2017; PINO *et al*., 2019).

A preocupação com a segurança do uso de probióticos cresce à medida que há, um aumento da oferta de alimentos suplementados com probióticos. A maioria dos probióticos é comercializada como gênero alimentício e não como produto farmacêutico ou biológico, mas é de extrema importância que a sua segurança seja levada em consideração. A segurança dos microrganismos tradicionalmente usados é confirmada pelo longo período de pesquisas, principalmente no trato gastrointestinal (JENSEN *et al*., 2012).

Os fatores que devem ser considerados na avaliação da segurança dos

probióticos incluem patogenicidade, infectividade, fatores de virulência compreendendo toxicidade, atividade metabólica e propriedades intrínsecas do microrganismo. *Bifidobacterium* são as bactérias predominantemente presentes no intestino de bebês durante a amamentação materna e são consideradas contribuintes da saúde deles. Estudos realizados com *Bifidobacterium* sugerem sua baixa patogenicidade, mas seu perfil de probiótico seguro pode estar relacionado ao número reduzido de estudos realizados com esta cepa (SILVA, 2016).

É importante destacar que os probióticos são seguros para o uso de pessoas saudáveis, mas devem ser administrados com cautela em pessoas debilitadas para evitar o risco de sepse. Embora ainda haja muito a ser estudado sobre os mecanismos de ação e as adequadas vias de administração dos probióticos, entende-se que diferentes cepas podem ter efeitos diferentes em pessoas saudáveis ou doentes, em diferentes estágios de determinadas doenças e em diferentes grupos etários (SCHNEIDER, 2016).

2. OBJETIVOS

2.1 Geral

Isolar e identificar, por meio de análises fenotípicas, bactérias ácido láticas com potencial probiótico a partir de amostras de queijos coloniais.

2.2 Específicos

- Caracterizar o potencial probiótico dos isolados de bactérias ácido láticas, pela capacidade de multiplicação à diferentes concentrações de cloreto de sódio (NaCl), fermentação de carboidratos e capacidade de multiplicação em diferentes temperaturas;
- Analisar o efeito antimicrobiano de isolados perante alguns patógenos

potenciais e definir um perfil de suscetibilidade dos isolados a diferentes tipos de antimicrobianos;

- Selecionar isolados com potencial probiótico para realizar testes *in vitro* de resistência às condições ácidas, a presença de sais biliares, e resistência ao trato gastrointestinal superior de forma simulada.

3. METODOLOGIA

3.1 Isolamento, seleção e caracterização das bactérias

No período de abril a junho de 2019, amostras de queijo colonial (n=10) foram adquiridas nos supermercados de Francisco Beltrão, município localizado no Sudoeste do Estado do Paraná. Do total de amostras adquiridas, quatro (n=4) foram produzidos em Francisco Beltrão, duas (n=2) no município de Verê, duas (n=2) no município de Planalto, uma (n=1) no município de Marmeleiro e uma (n=1) em Marechal Candido Rondon, todos municípios do Estado do Paraná. As amostras foram obtidas em condições usuais de embalagem e temperatura, acondicionadas em recipiente com isolamento térmico e encaminhadas ao Laboratório de Microbiologia do Centro de Ciências da Saúde da Universidade Estadual do Oeste do Paraná – UNIOESTE, Campus de Francisco Beltrão – PR, onde permaneceram sob refrigeração até o momento das análises microbiológicas.

Posteriormente, no mesmo dia em que os queijos coloniais foram adquiridos, alíquotas de 25 g de cada amostra foram pesadas e transferidas, assepticamente, para frascos contendo 225 mL de solução salina (0,85%, pH 7) esterilizada. Após diluição decimal seriada (10^{-1}, 10^{-2} e 10^{-3}), alíquotas de 100 µL foram plaqueadas em ágar De Man, Rogosa e Sharpe, (MRS, Neogen Corporation®) e incubadas a 37º C, em jarras de anaerobiose em microaerofilia com vela, durante 48 h. As colônias foram enumeradas e foram coletadas 10 colônias por amostra de queijo colonial que apresentaram morfotipos distintos e transferidas, individualmente para tubos de ensaio contendo 10 mL de caldo MRS e incubadas a 37°C, durante 24h, totalizando 100 isolados de BAL.

Os isolados foram conservados em caldo Brain Heart Infusion (BHI, Neogen Corporation®) + glicerol (25%) a -20°C para posterior caracterização.

3.2 Crescimento dos isolados para os diferentes testes

Os isolados foram cultivados em caldo BHI, durante 24h, a 37ºC e reinoculados sobre as mesmas condições, para todos os testes.

3.3 Caracterização fenotípica dos isolados bacterianos

Para a caracterização fenotípica os 100 isolados foram testados quanto a coloração diferencial de Gram e à reação de catalase. Apenas os isolados classificados como Gram-positivos e catalase negativos foram submetidos às análises posteriores, por serem consideradas características básicas de BAL.

Dando continuidade a caracterização das BAL foram realizados teste de capacidade de sobrevivência em diferentes temperaturas (10ºC e 45ºC) e a fermentação de diferentes tipos de carboidratos (glicose, lactose, sorbitol e manitol).

3.4 Capacidade de sobrevivência a diferentes temperaturas

Os isolados foram testados quanto à capacidade de se multiplicarem a 10°C e 45°C. Em tubos contendo 10 mL de caldo MRS foram inoculados individualmente com 100 µL de cultura ativa de diferentes isolados de BAL, em crescimento *overnight*. Os tubos foram incubados a 10 ºC ou a 45ºC por 48h. Como controle, foram utilizados os mesmos isolados de BAL cultivados em caldo MRS a 37ºC por 48h, em pH 7. O crescimento bacteriano foi verificado após 24 e 48h, comparando-se visualmente, o grau de turvação entre os tubos controle e teste. O experimento foi realizado em triplicata.

3.5 Fermentação de carboidratos

O perfil fermentativo dos isolados de BAL foi avaliado por meio da capacidade de fermentar os carboidratos glicose, lactose, sorbitol e manitol, com produção de gás. Em tubos contendo 10 mL de meio mínimo (10g de peptona bacteriológica

Kasvi®, 5g de NaCl Neon Comercial LTDA®, 0,3g de fosfato de potássio dibásico (K_2 HPO_4) (Vetec Química Fina®), 0,0018g de vermelho de fenol Vetec Química Fina®) e 5g do carboidrato a ser testado, autoclavados, foram inoculados individualmente com 100 µL de cultura ativa de diferentes isolados de BAL, incubadas a 37°C por 48h. Para observar o crescimento microbiano e a produção de gás, foram adicionados tubos de Durham invertidos aos tubos de cultura. Os isolados cujos tubos de Durham observou-se a produção de gás foram caracterizados como heterofermentativos (produzem ácido lático, dióxido de carbono, ácido acético, etanol, aldeído e diacetileno) e os isolados que turvaram o meio de cultura mas não produziram gás foram caracterizados como homofermentativos (produzem ácido lático). O experimento foi realizado em triplicata.

3.6 Capacidade de multiplicação a diferentes concentrações de NaCl

Os isolados foram testados quanto à capacidade de multiplicação a concentrações de 4% e 6,5% de NaCl (m/v). Tubos contendo 10 mL de caldo MRS adicionados de 4% ou 6,5% de NaCl foram inoculados individualmente, com 100 µL de cultura ativa de diferentes isolados de BAL. Os tubos foram incubados a 37º por 7 dias. Como controle, foram utilizados os mesmos isolados de BAL cultivados em caldo MRS, a 37ºC por sete dias, em pH 7, sem adição de NaCl. O crescimento bacteriano foi verificado a cada 24h, comparando-se visualmente, o grau de turvação entre os tubos controle e teste. O experimento foi realizado em triplicata.

3.7 Critérios para a seleção dos 20 isolados

A partir dos testes de coloração diferencial de Gram, catalase, resistência a diferentes temperaturas, resistência a diferentes concentrações de NaCl e fermentação de carboidratos foram selecionados 20 isolados de BAL para a realização dos demais testes. Como critérios de inclusão foram escolhidos dois isolados de cada queijo colonial, sendo eles: cocos ou bacilos, Gram-positivos, catalase negativos, fermentadores de todos os carboidratos testados, resistentes as diferentes concentrações de NaCl e ter se multiplicado nas diferentes temperaturas

testadas.

3.8 Atividade antimicrobiana das BAL

A capacidade inibitória das BAL foi verificada pela formação de halo de inibição sobre três microrganismos patogênicos: *Escherichia coli ATCC 25922*, *Staphylococcus aureus ATCC 25923* (cedidas pela Unisep - União de Ensino do Sudoeste do Paraná) e *Salmonella Typhimurium ATCC 14028* (cedidas pelo Instituto Oswaldo Cruz), pois no Brasil, a maioria das doenças transmitidas por alimentos de origem bacteriana são causadas principalmente por *Salmonella*, *Escherichia coli* e *Staphylococcus.*

Após a ativação dos 20 isolados de BAL, em caldo BHI por 24h a 37°C, foram inoculados 2 µL de cada cultura na superfície de placas de Petri contendo ágar MRS solidificado, em 5 pontos diferentes em cada placa, para que houvesse a formação de colônias. As placas foram incubadas a 37°C por 24h.

Os microrganismos patogênicos por sua vez, foram ativados em caldo BHI a 37°C por 24h. Alíquotas de 100 µL do meio de cultivo contendo os microrganismos patogênicos foram transferidas para tubos de ensaio contendo 10 mL de caldo BHI, onde realizou-se uma diluição seriada até 10^{-2} e em seguida, 750 µL do volume final foram transferidos para um tubo com 10 mL de BHI ágar a 0,87% (ágar semissólido), pré-preparado e mantido liquefeito em banho-maria a 45ºC. Seu conteúdo foi vertido sobre uma das placas de ágar MRS, onde havia sido feito a formação das colônias de BAL isoladas das amostras. Após solidificação da sobrecamada de ágar BHI semissólido, as placas foram reconduzidas à estufa de cultura onde permaneceram por mais 24 a 48h. A presença de halo de inibição no meio (≥5 milímetros de diâmetro) foi considerada indicadora da produção de substâncias inibitórias produzidas pelas BAL. O experimento foi realizado em duplicata.

3.9 Suscetibilidade antimicrobiana

A suscetibilidade a antimicrobianos foi avaliada pelo teste de difusão em ágar Müller-Hinton (MH), realizado de acordo com as normas do *Clinical and Laboratory Stardards Institute* (CLSI 2017). Após o cultivo em ágar MRS a 37ºC por 24h, preparou-se suspensão de colônias de BAL em solução salina esterilizada (0,85%)

até obter-se uma turvação equivalente a 0,5 da escala de MacFarland (1 x 10^6 UFC/mL^{-1}). Cada suspensão foi inoculada com o auxílio de um *swab* esterilizado na superfície de placas contendo Ágar Müller-Hinton. Após a secagem da superfície do ágar, adicionou-se assepticamente com o auxílio de uma pinça os discos de papel impregnados com os seguintes antimicrobianos: azitromicina (15µg), clindamicina (2µg), cloranfenicol (30µg), ampicilina (10µg), sulfazotrim (25µg), amoxicilina (10µg), eritromicina (15µg), levofloxacino (5µg), norfloxacino (10µg), amicacina (30µg). As placas com os antimicrobianos foram incubadas em estufa bacteriológica a 37ºC por 24h e os diâmetros das zonas de inibição foram medidos utilizando-se paquímetro. Como controle de qualidade foram utilizadas cepas padrão de *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 e *Salmonella* Typhimurium ATCC 14028. A leitura e a interpretação dos resultados foram realizadas de acordo com os padrões do *Clinical and Laboratory Standards Institute* (CLSI 2005). O experimento foi realizado em duplicata.

3.10 Tolerância das BAL a condições ácidas

A fim de analisar a tolerância às condições ácidas, 20 isolados de BAL foram inoculados em caldo BHI a 37°C por 24h. A resistência a diferentes condições ácidas foi testada em caldo MRS (pH 7), ajustado a pH 2, 3 e 4 com ácido clorídrico (HCl) concentrado, sendo que o pH 7 foi usado como controle. Tubos contendo 10 mL de caldo MRS acidificado foram inoculados com 100 µL de cultura ativa de diferentes isolados de BAL e incubados a 37ºC. Após exposições às condições ácidas de 0, 2 e 4h realizou-se diluições seriadas até 10^{-6} de cada tempo e 100 µL da diluição 10^{-6} foram inoculados em ágar, e as placas incubadas a 37°C durante 24h. Como controle, foram utilizados os mesmos isolados de BAL cultivados em caldo MRS, a 37ºC, em pH 7.

Posteriormente, foi realizada a contagem de células sobreviventes, expressa como valores de Unidades Formadoras de Colônias por mL (UFC.mL^{-1}). O experimento foi realizado em duplicata.

3.11 Resistência ao TGI superior de forma simulada

Após 24h de incubação em caldo BHI a 37ºC, os 20 isolados de BAL foram

separados por centrifugação (4000 x g por 5 minutos). Os pellets de células dos microrganismos isolados foram lavados duas vezes com tampão fosfato salina (0,85%) e ressuspendidas em 5mL de solução salina de NaCl a 0,5%. Uma alíquota de 200 µL da suspensão celular foi misturada a 300 µL de solução salina e 1mL de suco gástrico ou suco intestinal simulado e incubados a 37ºC por 48h. O suco gástrico simulado consistiu em pepsina (3mg.mL^{-1}) e pH 2 com ou sem a adição de leite integral estéril, reconstituído a 10% (m/v); enquanto o suco intestinal simulado foi composto por pancreatina (1mg.mL^{-1}), pH 8 com ou sem adição de 0,5% de sais biliares. O efeito da presença de um alimento na sobrevivência durante o trânsito gástrico em pH 2 foi avaliado da mesma forma, porém, substituindo a solução salina (0,85%) por 300 µL de leite integral reconstituído a 10% (m/v). A contagem do número de células viáveis durante a simulação de passagem pelo trato gástrico e pelo trato intestinal foi realizada nos tempos 0, 90 e 240 min, plaqueando 100 µL da cultura em placas de Petri contendo ágar MRS. Os dados foram expressos como valores de UFC.mL^{-1}. O experimento foi realizado em duplicata.

3.12 Identificação das espécies

Os 20 isolados dos queijos coloniais foram submetidos ao teste de identificação microbiológica através do sistema Matrix Associated Laser Desorption-Ionization - Time of Flight (MALDI-TOF). Os isolados testados foram semeados pela técnica de esgotamento em ágar MRS de forma a obter colônias isoladas e foram enviadas via Sedex-Correios, para o laboratório AQUACEN da Escola de Veterinária da Universidade Federal de Minas Gerais (UFMG) para a identificação dos isolados a partir da metodologia de MALDI –TOF.

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5. SELECTION OF LACTIC ACID BACTERIA WITH PROBIOTIC POTENTIAL FROM COLONIAL CHEESE

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Sentence abstract: Ten samples of colonial cheese were analyzed in order to isolate and identify, through phenotypic analyzes, the probiotic potential of lactic acid bacteria.

Abstract

Handmade cheeses have a diverse microbial population, among them are lactic acid bacteria. In this context, the present study aimed to isolate and identify, through physiological analyzes, lactic acid bacteria with probiotic potential from samples of colonial cheeses. For that, ten samples of colonial cheese were used to isolate bacterial cultures. The bacteria were phenotypically characterized and tested for resistance to different temperatures, carbohydrate fermentation capacity and growth capacity at different NaCl concentrations. Subsequently, were selected 20 isolates for analysis of activity and antimicrobial susceptibility, tolerance and resistance to acidic environment. It was observed that the majority of the bacteria presented gram-positive and catalase-negative bacilli, all of them showed growth at the temperatures evaluated (10 ° C and 45 ° C) and most fermented all carbohydrates (glucose, lactose, sorbitol and mannitol) with gas production, characterized as heterofermentative. Regarding resistance to different antimicrobials, 75% of the isolates were resistant to 2 or more antimicrobials. The isolates also showed little sensitivity to the acidic environment, with a longer survival time when the acidic environment was associated with milk and excellent resistance in intestinal conditions. The isolates identification by Maldi tof identified five as *Lactobacillus brevis*, two as *Enterococcus faecium*, three as *Pediococcus acidilactici* and one as *Lactobacillus rhamnosus*. In this context, the BAL evaluated have an important

probiotic potential, which deserves to be highlighted in future research, since they have positive aspects in relation to the evaluated items.

Keywords: Microorganisms, phenotypic characterization, microbiota, fermented foods, antagonism.

Introduction

Handmade cheeses, popularly known as colonial cheeses, are commonly produced in the Southwest region of the State of Paraná. The production techniques are transmitted verbally a from generation to generation. Due to the handling techniques and, since they are produced, in most cases, with raw milk and without the addition of an initial inoculum, colonial cheeses have a diversified unwanted microbial population, which presents itself as a factor of deterioration of the product and microbiological danger to consumers (Hermanns 2013; Pehrson 2017).

In addition to these undesirable microorganisms, lactic acid bacteria (LAB) are naturally present in these cheeses, which can present themselves in the form of cocci or Gram-positive bacilli, are catalase-negative, non-spore-forming, phylogenetically distinct, immobile, facultative anaerobic, able to carry out fermentation in anaerobiosis, as well as in aerobiosis, but in a slower way (Salminen et al. 1998; Carr, Chill and Maida 2002). LABs are essential for the fermentation process and one of the oldest forms of preservation, due to the reduction of pH and consequent production of organic acids, such as lactic acid, from the fermentation of available carbohydrates (CHO), becoming the main antagonistic effect against different microorganisms (Corbo et al. 2009; Hermanns 2013).

Aiming at the probiotic potential, the LAB have been incorporated into cheeses, as they have adequate characteristics, such as their buffering capacity and high fat content, offering protection to bacteria during the passage through the gastrointestinal tract (GI tract) (Back et al. 2013). Among the benefits generated by the probiotic effect of LAB can be highlighted the antimicrobial and antioxidant activity, the intestinal infections control, the improvement in the absorption of some nutrients, a better use of lactose and relief of the symptoms of intolerance to this sugar, the cholesterol levels reduction, the anticarcinogenic effect and the increased immune response due to the production of antibodies (Saarela et al. 2002; Vasiljevic

and Shah 2008; Back et al. 2013).

Considering the important role of LAB on the human organism, the need to develop studies that are capable of characterizing the bacterial colonization of colonial cheeses, especially of these bacteria. In order to encourage the consumption of regional products, as well as to favor the local economy. Thus, the aim of the present study was to isolate and identify, through phenotypic analyzes, LAB with probiotic potential from colonial cheeses samples.

Materials and methods

Isolation, selection and characterization of bacteria

In the period from April to June 2019, 10 samples of colonial cheese were purchased from Francisco Beltrão supermarkets, a city located in the southwest of Paraná state. The samples were obtained under usual packaging conditions. They were placed in a thermally insulated container and sent to the Microbiology Laboratory, in the Health Sciences Center from the State University of Western Paraná - UNIOESTE, Francisco Beltrão Campus - PR. Where they remained refrigerated until the moment of microbiological analyzes.

After that, 25 g aliquots of each colonial cheese were weighed and transferred, aseptically, to vials containing 225 mL of sterile saline solution (0.85%, pH 7). After the serial decimal dilution (10^{-1}, 10^{-2} e 10^{-3}), a 100 µL aliquots were plated on agar De Man, Rogosa e Sharpe (MRS, Neogen Corporation®) and incubated at 37 ° C, in anaerobic jars, for 48 h until the colonies formation. The colonies were listed and 10 colonies from each sample of colonial cheese that presented distinct morphotypes were collected and transferred individually to MRS broth in test tubes and incubated at 37°C for 24 hours, totaling 100 LAB isolates.

After that, the isolates were preserved in Brain Heart Infusion (BHI) + glycerol (25%) a -20°C for further characterization.

Growth of isolates for different tests

The isolates were grown in BHI broth, for 24h, at 37ºC and re-inoculated under the same conditions, for all tests.

Phenotypic characterization of bacterial isolates

For the phenotypic characterization, the 100 were tested for Gram stain and catalase reaction. Only isolates classified as Gram-positive and catalase-negative were subjected to further analysis, as they are considered basic characteristics of LAB.

Continuing characterization of LAB growth tests were carried out at different temperatures (10ºC and 45ºC) and fermentation in different types of carbohydrates (glucose, lactose, sorbitol and mannitol).

Capability to survive at different temperatures

The isolates were tested for their capability to multiply at 10°C and 45°C. In tubes containing 10 mL of MRS broth, they were inoculated with 100 µL of active culture from different LAB isolates. The tubes were incubated at 10ºC or 45ºC for 48h. As a control, the same LAB isolates were grown in MRS broth at 37ºC for 48h, at pH 7. The bacterial growth was verified after 24 and 48h. It was visually compared the turbidity degree between the control and test tubes. The experiment has been carried out in triplicate.

Carbohydrate fermentation

The fermentative profile of the LAB isolates was evaluated by the capability to ferment the carbohydrates glucose, lactose, sorbitol and mannitol, with gas production. In tubes containing 10 ml of minimal medium (10g of Kasvi® bacteriological peptone, 5g of NaCl Neon Comercial LTDA®, 0.3g of dibasic potassium phosphate (K2 HPO4) Vetec Química Fina®, 0.0018g of phenol red Vetec Química Fina®) and 5g of the carbohydrate to be tested were inoculated with 100 µL of active culture from different LAB isolates, incubated at 37°C for 48h. To observe microbial growth and gas production, inverted Durhan tubes were added into the culture tubes. The isolates in which the tubes were observed gas production were characterized as heterofermentativos (they produce lactic acid, carbon dioxide, acetic acid, ethanol, aldehyde and diacetylene) and the isolates that did not produce gas were characterized as homofermentative (they produce lactic acid). The experiment was carried out in triplicate.

Growth capacity at different NaCl concentrations

The isolates were tested for growth capacity at concentrations of 4% and 6.5% NaCl. In tubes containing 10 mL of MRS broth added with 4% or 6.5% NaCl were inoculated with 100 µL of active culture from different LAB isolates. The tubes were incubated at 37ºC for 7 days. As a control, the same LAB isolates were grown in MRS broth, at 37ºC, for 7 days, at pH 7, without addiction of NaCl. Bacterial growth was checked every 24 hours, visually comparing the degree of turbidity between the control and test tubes. The experiment was carried out in triplicate.

Criteria selection for the 20 isolates

From the Gram stain tests, catalase tests, temperatures resistance, resistance to different concentrations of NaCl and fermentation of carbohydrates 20 isolates of LAB were selected to perform the other tests. As inclusion criteria, two isolates from each colonial cheese were chosen. They are cocci or bacilli, Gram-positive, catalase-negative, fermenters of all tested carbohydrates, resistant to different concentrations of NaCl and they multiplied at the different temperatures tested.

LAB antimicrobial activity

The inhibitory activity of LAB was verified by the formation of an inhibition halo on the indicator microorganisms: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 (provided by Unisep - Teaching Union of the Southwest of Paraná) and *Salmonella* Typhimurium ATCC 14028 (provided by Oswaldo Cruz Institute).

After activation of the 20 BAL isolates, in BHI broth for 24h at 37 ° C, 2µL of each culture was inoculated on the plate surface containing MRS agar, 2 µL of each culture, at 5 different points on each plate, so that colonies were formed. The Petri dishes were incubated at 37°C for 24h.

The indicator microorganisms were activated in BHI broth at 37°C for 24 hours. Aliquots of 100 µL of the culture medium containing the indicator microorganisms were transferred to test tubes with 10 mL of BHI broth. Where a serial dilution up until 10^{-2} was performed and then 750 µL of the final volume was pipetted and transferred to a tube with 10 mL of BHI 0.87% agar (semi-solid agar), pre-prepared and kept liquefied in a water bath at 45ºC. Its contents were transferred into the MRS agar plates, where the LAB colonies isolated from samples had been

formed.

After complete solidification of the BHI semi-solid agar overlay, the plates were returned to the culture oven, where they remained for another 24 to 48 hours. The presence of an inhibition halo in the culture medium (≥5 mm - mm) was considered an indicator of the production of inhibitory substances produced by LAB. The experiment was carried out in duplicate.

Antimicrobial susceptibility

Susceptibility to antimicrobials was assessed by the diffusion test on Müller-Hinton agar (MHA), carried out according to the standards of the *Clinical and Laboratory Stardards Institute* (CLSI 2017). After cultivation on MRS agar at 37ºC for 24h, The LAB colonies were suspended in a sterile saline solution (0.85%) until a turbidity compatible with the 0.5 degree of the MacFarland scale (1 x 10^6 CFU/mL) was obtained. Each suspension was inoculated with the aid of a *swab* on the surface of plates containing MHA. After drying the agar surface, paper discs were aseptically added with the aid of tweezers. They were impregnated with the following antimicrobials: azithromycin (15µg), clindamycin (2µg), chloramphenicol (30µg), ampicillin (10µg), sulfazotrim (25µg), amoxicillin (10µg), erythromycin (15µg), levofloxacin (5µg), norfloxacin (10µg), amikacin (30µg). The plates with the antimicrobials were incubated in a bacteriological oven at 37ºC for 24h. The zones of inhibition diameters were measured using a caliper. The experiment was carried out in duplicate.

LAB tolerance to acidic conditions

In order to analyze tolerance to acidic conditions, LAB isolates were inoculated in BHI broth at 37°C for 24h. Resistance to different acidic conditions was tested in MRS broth (pH 7), adjusted to pH 2, 3 and 4, with concentrated hydrochloric acid (HCl), pH 7 was used as a control. Tubes containing 10 mL of acidified MRS broth were inoculated with 100 µL of active culture from different LAB isolates and incubated at 37ºC. After exposure to acidic conditions of 0, 2 and 4h, serial dilutions were made up until 10^{-6} of each time period. And 100 µL of the 10^{-6} dilution were plated on agar and the plates were incubated at 37°C for 24h. As a control, the same LAB isolates were cultivated in MRS broth, at 37ºC for 24h and pH 7.

Subsequently, cell survival was counted and expressed using logarithmic

notation, as Colony-forming unit per mL (CFU.mL^{-1}).

Simulated upper gastrointestinal tract resistance

After 24h of incubation in BHI broth at 37ºC, the LAB isolates were separated by centrifugation (4000 x g for 5 minutes). The pellet cells were washed twice with phosphate buffered saline (0.85%) and resuspended in 5mL of 0.5% saline solution. An aliquot of 200 µL of the cell suspension was mixed with 300 µL of saline solution and 1 ml of gastric juice or simulated intestinal juice and incubated at 37ºC for 48 hours. The simulated gastric juice consisted of pepsin (3mg.mL^{-1}) and pH 2 with and without the addition of whole milk; the simulated intestinal juice was composed of pancreatin (1mg.mL^{-1}), pH 8 with and without the addition of 0.5% bile salts. The presence effect of a food on survival during gastric transit at pH 2 was evaluated in the same way, however, replacing the saline solution (0.85%) with 300 µL of reconstituted whole milk at 10% (m/v). The counting of viable cells during the simulation by the gastric and intestinal tracts was performed at times 0, 90 and 240 minutes, plating 100 µL of the culture in petri dishes containing MRS agar. The data were expressed as CFU.mL^{-1} values. The experiment was carried out in duplicate.

Species identification

The 20 isolates from colonial cheeses were submmited to the microbiological identification test using the system Matrix Associated Laser Desorption-Ionization - Time of Flight (MALDI-TOF). The samples were sown by the depletion technique on MRS agar in order to obtain isolated colonies, after that, they were immediately sent via Sedex to the AQUACEN laboratory, from Veterinary School of Federal University of Minas Gerais (UFMG) for the identification of the isolates by using the MALDI – TOF methodology.

Results

The total of 100 LAB isolates analyzed, 67% were bacilli, 100% of Gram-positive isolates and 97% negative (97%), characteristic from bacteria to the genus *Lactobacillus* spp. In relation to the multiplication capacity at different temperatures, all isolates have developed at 10°C and 45°C after 48 hours of incubation. Regarding the ferment carbohydrates capability, it was found that 93% of the tested isolates

fermented glucose, 99% mannitol and gas formation, and all fermented lactose and sorbitol with gas production, what characterized them as heterofermentative.

All isolates when exposed to different concentrations of NaCl (4% and 6.5%) were able to multiply. The turbidity and cell mass deposit was being observed at the bottom of all tubes, when compared to control tubes.

After the preliminary tests, 20 isolates were selected to continue the following tests. Regarding antimicrobial activity (Table 1), it was observed that 45% of the LAB isolates showed inhibition of the pathogen *Staphylococcus aureus* (1, 6, 7, 8, 10, 12, 13, 14, 18), 65% inhibited *Escherichia coli* (1, 2, 3, 8, 9, 10, 11, 12, 15, 16, 18, 19, 20) and 60% inhibited *Salmonella Typhimurium* (1, 2, 4, 5, 6, 8, 10, 12, 13, 14, 17, 18).

Table 1. Analysis of the antimicrobial activity of LAB isolated from colonial cheese, expressing the Mean ± Standard Deviation of the inhibition halos in millimeters.

Isolated	***Staphylococcus aureus***	***Escherichia coli***	***Salmonella* Typhimurium**
1	13,6±1,15	13,1±3,32	11,5±3,40
2	-	26,2± 3,54	13,8±1,93
3	-	12,5±1,67	-
4	-	-	19,5±3,16
5	-	-	15,8±1,11
6	13,7± 3,54	-	14,9±2,95
7	12,1±1,90	-	-
8	16, ±7,07	19,3±3,29	-
9	-	1,74±2,05	-
10	13,9±1,01	15,9±3,46	11,1±0,86
11	-	13,5±2,75	-
12	15,4±3,26	13,2±3,35	13,4±4,27
13	13,6±1,15	-	10,6±1,00
14	16,2±3,70	-	27,0±5,05
15	-	12,5±3,34	-
16	-	12,6±2,35	-
17	-	-	17,6±4,02

18	11,7±1,88	17,1±4,01	10,8±1,73
19	-	13,1±3,32	-
20	-	15,7±5,23	-

The antimicrobial susceptibility test (Table 2) demonstrated that the strains of *Lactobacillus spp*. were resistant to azithromycin (55%), clindamycin (85%), chloramphenicol (15%), ampicillin (40%), sulfazotrin (95%), amoxicillin (25%), erythromycin (20%), levofloxacin (15 %), norfloxacin (10%), amikacin (30%). In contrast, some isolates were sensitive to the antimicrobial azithromycin (40%), chloramphenicol (85%), ampicillin (40%), sulfazotrin (5%), amoxicillin (75%), erythromycin (80%), levofloxacin (15 %), norfloxacin (10%), amikacin (30%), and none of the isolates showed sensitivity to clindamycin. Furthermore, the strains showed intermediate resistance only to azithromycin (5%) (Table 2).

Regarding the different classes of antimicrobials tested, all had 2 or more resistant isolates. The beta-lactams class and sulfonamides were the ones that showed the greatest resistance of the isolates. Both classes obtained a total of 95% of the isolates resistant to at least one of the class antimicrobials. The lincosamides that showed a total of 85% of resistant isolates, and the macrolides class that added 75% of isolates resistant to their antimicrobials. In contrast, the quinolones class showed only 25% of resistant isolates, followed by the class of amphenicols, which showed the least resistance on the part of isolates, only 15% resistant.

Of the total isolates tested, 75% have showed a multidrug resistance profile, showing resistance to three or more different classes of antimicrobials. On the other hand, isolate 15 was not resistant to any of the tested antibiotics, isolate 18 was resistant only to the sulfonamide class, isolate 19 was resistant to sulfonamides and macrolides, and isolates 5 and 11 were resistant only to the sulfonamide class and lincosamides.

Table 2. Result regarding the resistance of LAB strains isolated in the study to different types of antimicrobials.

Isolated	Antimicrobials tested									
	AZI	CLI	CLO	AMP	SUT	AMO	ERI	LEVO	NORF	AMI
1	R	R	S	S	R	S	S	R	S	R
2	I	R	S	R	R	R	R	S	S	R
3	R	R	S	R	R	R	R	S	S	S
4	S	R	S	R	R	S	S	S	S	S
5	S	R	S	S	R	S	S	S	S	S
6	R	R	R	R	R	R	R	S	S	S
7	S	R	S	S	R	S	S	S	R	S
8	R	R	S	S	R	S	S	S	S	R
9	S	R	S	R	R	S	S	S	I	R
10	R	R	S	R	R	S	S	R	R	R
11	S	R	S	S	R	S	S	S	S	S
12	R	R	R	R	R	R	S	S	S	S
13	R	R	R	S	R	S	S	S	S	I
14	S	R	S	R	R	R	R	S	S	S
15	S	I	S	S	S	S	S	S	S	I
16	R	R	S	S	R	S	S	R	S	S
17	R	R	S	S	R	S	S	S	S	S
18	S	I	S	S	R	S	S	S	S	S
19	R	I	S	S	R	S	S	S	I	S
20	R	R	S	S	R	S	S	S	S	R

Note: AZI: Azithromycin; CLI: Clindamycin; CLO: Chloramphenicol; AMP: Ampicillin; SUT: Sulfazothrin; AMO: Amoxicilin; ERI: Erythromycin; LEVO: Levofloxacin; NORF: Norfloxacin; AMI: Amikacin.

R: Resistant; S: Sensitive; I: Intermediate.

The analysis of the tolerance of the 20 isolates to acidic conditions showed that the isolates 1, 3, 4, 5, 6, 7, 8, 9, 15, and 20 showed survival at all times for media with pH 2, 3 and 4. Microorganisms 2, 10, 11, 12, 13, 14, 16, 17, 18 and 19 survived at all times for media with pH 3 and 4. At pH 2 they only survived at 0 and 2h. Isolate 13 survived at all times for media with pH 3 and 4, and at pH 2 only at 0h (Table 3).

Table 3. Final counts of viable microorganisms of the 20 strains submitted to cultivation at different pH values for 48 hours.

Isolated	Tested times	Final countdown (N x 10^{-9} CFU.mL)			
		pH 7	pH 2	pH 3	pH 4
	0h	2,7	2,0	2,5	2,0
1	2h	2,8	1,5	2,0	1,5
	4h	3,5	9,0	2,2	3,0
	0h	4,0	1,2	3,1	3,5
2	2h	3,7	1,4	2,1	3,1
	4h	4,1	-	2,6	3,1
	0h	3,1	2,3	2,1	2,7
3	2h	2,9	2,6	3,3	1,8
	4h	3,6	7,0	3,8	3,6
	0h	3,0	3,3	3,2	4,2
4	2h	2,7	2,0	2,3	2,9
	4h	3,8	2,5	2,5	2,9
	0h	1,4	3,4	1,8	4,0
5	2h	3,3	6,4	3,2	3,5
	4h	3,7	2,6	2,7	4,2
	0h	3,4	1,6	2,9	3,8
6	2h	3,3	3,9	2,1	4,4
	4h	3,8	2,0	1,6	3,3
	0h	3,0	6,2	4,1	3,8
7	2h	3,9	3,9	3,1	2,1
	4h	4,3	2,0	4,2	1,9
	0h	2,6	7,1	1,7	1,9
8	2h	4,7	6,5	1,9	1,9
	4h	4,9	1,7	2,2	2,0
	0h	3,2	2,0	3,1	4,9
9	2h	4,3	1,6	2,0	2,2
	4h	4,9	1,3	8,6	3,1
10	0h	3,3	7,0	6,4	2,3

	2h	4,0	5,1	8,1	3,0
	4h	4,3	1,9	7,5	3,7
	0h	1,9	4,2	3,0	4,0
11	2h	2,1	2,0	1,7	2,6
	4h	3,9	-	2,0	3,1
	0h	4,7	3,2	1,3	8,3
12	2h	1,2	2,0	6,1	1,2
	4h	2,3	-	5,5	1,9
	0h	2,4	2,4	6,3	6,3
13	2h	3,9	-	1,9	1,7
	4h	9,9	-	2,0	3,0
	0h	1,7	1,9	4,4	6,2
14	2h	3,8	1,0	4,0	3,6
	4h	5,9	-	3,3	2,5
	0h	4,6	1,1	3,6	2,0
15	2h	5,4	7,0	7,0	1,5
	4h	6,1	5,0	4,0	1,3
	0h	4,0	5,2	4,7	3,8
16	2h	1,5	2,0	1,1	3,9
	4h	2,6	-	5,2	1,0
	0h	2,1	2,1	5,0	4,8
17	2h	2,2	8,0	7,1	3,4
	4h	2,1	-	5,0	1,1
	0h	2,1	2,2	4,0	6,1
18	2h	1,2	3,0	7,5	1,5
	4h	1,4	-	5,0	1,4
	0h	1,1	1,6	2,4	3,0
19	2h	2,2	2,0	5,0	1,1
	4h	1,3	-	1,9	1,3
	0h	2,4	2,4	1,0	1,9
20	2h	7,2	3,3	4,3	3,0
	4h	6,1	1,0	7,5	8,0

Note: CFU = Colony-Forming Unit; N: Values obtained.

When applied to the tolerance analysis of gastric juice simulated with pepsin (pH 2), all 20 isolates decreased the number of CFU. mL^{-1} as the stipulated time 0, 2 and 4 hours passed. The microorganisms 3, 4, 6, 7, 9, 11, 12, 13, 14, 15, 16, 18 and 20 survived at all analyzed times, even though their colonies number have decreased considerably over the time In contrast, isolates 2, 5, 8, 10, 17 and 19 survived only at 0 and 1h30min, with no colony remaining after 4h, so that isolate 1 survived only at time 0h (Table 4). In the tolerance test of gastric juice simulated with pepsin pH 2 add to the milk, it was observed that all microorganisms showed growth at 0, 1h30min and 4h, with values of CFU.mL^{-1} similar to the control microorganisms (MRS broth, pH 7) (Table 4).

Considering the tolerance analysis to simulated intestinal juice, with pancreatin pH 8, and pancreatin pH 8 + 0.5% bile salts, the isolates have showed growth in both treatments and at all times analyzed (0, 1h30min and 4h). As the hours passed the number of CFU. mL^{-1} increased in all microorganisms, similar to those presented by the isolated controls (Table 4).

Table 4. Final counts of viable microorganisms from the 20 strains subjected to cultivation for 48 hours under different conditions to simulate in vitro resistance to conditions similar to the gastrointestinal tract.

Isolated	Final countdown (N x 10^{-9} CFU.mL)											
	Pure pepsin pH 2			Pepsin + milk pH 2			Pure pancreatin pH 8			Pancreatin + Bile bovine pH 8		
	0min	90min	240min	0min	90min	240min	0min	90min	240min	0min	90min	240min
1	3,9	-	-	2,2	3,3	2,7	2,5	1,5	1,3	2,4	3,5	2,8
2	3,2	1,1	-	2,7	1,9	1,5	1,9	3,0	3,8	3,8	3,2	3,6
3	2,9	1,4	8,0	2,8	1,9	3,9	1,9	9,0	5,7	3,0	3,8	4,0
4	2,4	2,1	2,0	1,3	2,7	1,9	1,2	8,2	4,7	2,4	3,5	2,8
5	2,4	2,1	-	1,0	1,7	1,8	1,4	1,3	1,0	2,9	1,7	3,4
6	1,3	7,5	2,0	8,2	1,4	1,4	2,1	1,8	2,7	1,7	1,4	1,5
7	2,3	2,0	2,0	7,7	9,0	9,5	1,3	1,6	2,4	1,0	7,2	7,0
8	2,2	1,2	-	1,2	3,2	9,2	2,6	2,1	3,1	9,0	7,0	8,0
9	3,3	7,5	3,0	1,0	1,7	2,2	9,5	8,5	1,1	1,9	1,2	1,4
10	2,9	1,6	-	1,6	2,6	9,0	1,3	1,7	1,1	1,5	1,4	1,3
11	3,6	3,3	1,4	1,0	1,1	1,3	9,0	9,7	2,4	9,0	1,0	1,2
12	7,0	7,5	3,5	7,0	9,5	1,3	7,0	7,7	9,0	6,2	7,5	1,5
13	3,3	4,0	3,0	4,0	1,0	1,3	4,0	6,5	9,5	1,0	1,7	1,9
14	8,5	2,7	2,5	9,0	1,0	1,0	1,3	1,8	2,0	3,5	1,7	1,9
15	5,2	5,0	1,5	2,0	7,0	7,1	2,5	5,0	7,5	1,5	3,5	6,2
16	2,8	1,1	1,0	2,7	2,5	9,0	1,5	4,0	9,0	2,5	3,6	5,7
17	6,0	3,6	-	4,0	11,0	7,0	5,0	4,0	8,7	2,0	1,5	4,0
18	4,0	8,0	2,0	1,7	6,5	7,3	5,0	7,0	8,5	9,0	3,0	6,2
19	3,2	1,1	-	7,5	5,2	5,5	9,0	1,3	1,9	8,7	1,2	1,9
20	3,2	7,0	1,0	4,0	1,1	1,5	4,5	8,5	10,5	2,2	1,2	1,1

Note: CFU = Colony-Forming Unit; N: Values obtained.

Through molecular analysis, isolates 1, 5, 6, 7 and 16 were identified as *Lactobacillus brevis*. Isolates 2 and 9 as *Enterococcus faecium*. Isolates 8, 11 and 13 as *Pediococcus acidilactici*, and isolate 19 as *Lactobacillus rhamnosus*.

Discussion

As Almeida Júnior (2015) and Uecker (2018) observed, when evaluating the presence of LAB in milk and dairy products, it was observed that the isolates had morphology of bacilli and cocci, they were Gram-positive and catalase negative. In the present study, the LAB were fermenters of different CHO and by they form gases they were considered heterofermentative. This information was different from Hermanns (2013) and Uecker (2018) who found that the LAB analyzed in milk and dairy products and artisanal cheeses, respectively, were homofermentative, since even though the environment was cloudy, they did not produce gas. It is important to highlight that homofermentative bacteria, from glucose fermentation, produce only lactic acid, while heterofermentative bacteria produce in addition to lactic acid, other compounds carbon dioxide, acetic acid, ethanol, aldehyde and diacetylene, which can contribute to the flavor and aroma characteristics of fermented dairy products (Carr et al. 2002; Jay 2005; Hermanns 2013).

When evaluating the multiplication capacity at different temperatures and the tolerance to different concentrations of NaCl, all isolates showed multiplication capacity, as well as in the study by Funck (2016) which has evaluated the probiotic, technological characteristics and safety aspects of Lactobacillus curvatus P99. The author observed that the microorganism also multiplied at temperatures of 10ºC and 45 ° C, as well as at NaCl concentrations of 4.5 and 6%, suggesting that it can survive food maturation processes fermented products such as cheese and salami.

Almeida Júnior (2015) when selecting LAB of artisanal goat cheese and autochthonous milk found that all isolates were tolerant to the concentration of 4% and 6.5% NaCl, as showed in this study. This characteristic is fundamental in the industrial application of LAB, especially in the cheese fermentation process, since these microorganisms must tolerate and remain viable to stressful conditions, such as acidity, temperature, salinity and freeze drying (Bremer and Kramer 2000).

LAB have been used in foods as natural preservatives, due to their power to inhibit several deteriorating and pathogenic microorganisms. In this study, the LAB

have showed a positive effect on inhibiting of *Staphylococcus aureus*, *Escherichia coli* e *Salmonella Typhimurium.* While Guedes Neto et al. (2005) when evaluating the antimicrobial activity of LAB isolated from artisanal and industrial rennet cheese also observed that the *Lactobacillus spp*. tested were effective in inhibiting strains of *Staphylococcus spp*. and *Escherichia coli* CM2M17.

Silva (2011) also has found positive antimicrobial effects of LAB against *Listeria monocytogenes* and *Staphylococcus aureus.* Hermanns (2013) observed important action by LAB against *Escherichia coli* ATCC 8739, *Listeria monocytogenes* ATCC 7466, *Staphylococcus aureus* ATCC 1901 and *Salmonella Typhimurium* ATCC 13076.

In contrast, Hartmann, Wilke and Erdmann (2011) observed in their study that *Lactobacillus curvatus* was able to inhibit *Listeria monocytogenes*, but did not inhibit *Bacilus cereus*, *Staphylococcus aureus* and *Salmonella enterica*. It is possible to conclude that the antimicrobial substances produced are bacteriocins, which has attracted attention due to their probiotic role, status GRAS (*Generally Recognized as Safe*) and its potential use as a safe additive in food preservation, which may inhibit the growth of pathogenic Gram-positive bacteria, yeasts and some species of Gram-negative bacteria (Dhewa 2012). As well as, the production of organic acids, hydrogen peroxide and substances with bactericidal or bacteriostatic actions, can also happen during lactic fermentation. So that, they can exert antagonistic activity against the growth of pathogenic and deteriorating bacteria in food. For this reason, there is a great interest in the use of LAB in foods with ingredients that favor the human and animal microbiota (Pan et al. 2009; Darsanaki et al. 2012).

Antimicrobial resistance is an increasingly frequent problem with worldwide spread, compromising the clinical treatment of various pathologies that affect humans and animals. Thus, LAB have been evaluated in order to be used as probiotics in the reconstitution of the intestinal microbiota (Silva 2011; Vitola et al. 2016), as well as a constant demand from the food industry for LAB that produce substances with antimicrobial potential, such as antimicrobial peptides, which inhibit various pathogenic and deteriorating microorganisms (Castellano *et al.* 2017).

Antimicrobial resistance is closely related to food safety and should always be investigated when there is an intention to use new strains of microorganisms in food products. Since these new strains can carry resistance genes that can be transferred to other bacteria, therefore, increase the potential for virulence and

present resistance to different antimicrobials, which can endanger human health (FAO/WHO, 2006).

As in the present study, some LAB showed resistance or sensitivity to antimicrobials, Funck (2016) and Vitola *et al.* (2016) also had the same evaluation, they verified that the isolates showed phenotypic resistance to ciprofloxacin, trimethoprim-sulfamethoxazole, sulfonamide, vancomycin, ampicillin and gentamicin; were sensitive to amikacin, cephalothin, ciprofloxacin, clindamycin, chloramphenicol, erythromycin, streptomycin, gentamicin penicillin and tetracycline.

In addition to the characteristics already discussed, the capability to survive in the environment in which it will act is an essential characteristic when choosing a probiotic microorganism (Maragkoudakis *et al*. 2006; Liu *et al*. 2013). In order to survive in the intestine, microorganisms must tolerate the action of digestive enzymes and the low pH of the stomach, ranging from 2.5 to 3.5, reaching pH 1.5 during fasting or 4.5 when the individual is fed; and digestive enzymes. This high degree of acidity can lead to the destruction of several microorganisms ingested, since most of them are sensitive to pH values below 3. However, it is important to highlight that the nature of the food is capable of altering the transit time in the gastrointestinal tract, which ussualy takes from 2h to 4h, enabling the microorganism to remain, due to its buffering and protective effect (Huang e Adams 2004; Huang *et al.* 2014).

Thus, the present study found that acidic conditions are capable of interfering in the LAB activity, while the presence of food (in this case, the milk) was able to maintain its viability. Meira (2010), Ranadheera *et al.* (2014), Funck (2016) and Uecker (2018) also observed similar results. Huang e Adams (2004) claim that the low tolerance of some strains when subjected to simulated gastric juice is not sufficient to remove their probiotic effect, since the strains can reach the intestine in high concentrations when buffered by food or encapsulated, thus promoting positive effects on human health.

It is worth mentioning that, for the product to be commercialized in Brazil with the probiotic property, the microorganisms used must have tolerance against the barriers of the gastrointestinal tract, as well as they must have a viable cells number sufficient to perform the beneficial functions to the human organism (Brasil 2008).

Similar to the present study, in which MALDI-TOF was used to identify the microorganisms present in the evaluated cheeses, Angeleti *et al*. (1998) used the

same method in the quality control of buffalo mozzarella cheese. Moreover, Kanak and Yilmaz (2019) used the method in the identification and detection of the antimicrobial activity of lactic acid bacteria isolated from local cheeses. It is important to highlight that in addition to its use in the detection of microorganisms, MALDI-TOF can be applied in the analysis of lysozyme present in cheese and in the identification of the lipid profile of cheese (Schneider, Becker and Pischetsrieder 2010; Damário *et al*. 2015).

By this context, it is possible to conclude that the LAB present in colonial cheeses marketed in a city located in the Southwest of Paraná State have probiotic potential, deserving prominence in future research, since they have positive aspects in relation to the evaluated items.

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