LUARA APARECIDA SIMÕES

ISOLAMENTO E POTENCIAL PROBIÓTICO DE BACTÉRIAS DO ÁCIDO LÁTICO E LEVEDURAS ISOLADAS DE AZEITONAS DE MESA BRASILEIRAS

LAVRAS – MG

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Orientador

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ISOLATION AND PROBIOTIC POTENTIAL OF LACTIC ACID BACTERIA AND YEASTS ISOLATED FROM BRAZILIAN TABLE OLIVES

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do programa de Pós Graduação em Microbiologia Agrícola, para obtenção do título de Doutor.

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RESUMO

As azeitonas de mesa são um produto fermentado obtido a partir dos frutos da oliveira (*Olea europaea* L.*)*, que podem conter microrganismos com propriedades funcionais. Este trabalho avaliou a diversidade microbiana e características físico-químicas de frutos frescos e durante a fermentação de azeitonas de mesa brasileiras e selecionou microrganismos potencialmente probióticos. Leveduras, bactérias mesófilas e bactérias do ácido lático (BAL) foram isoladas de azeitonas das cultivares Grappolo 541 e Ascolano, protegidas pela Empresa de Pesquisa Agropecuária de Minas Gerais (EPAMIG) e mantidas em coleção na Fazenda Experimental de Maria da Fé – Minas Gerais- Brasil. Os isolados foram identificados por técnicas polifásicas: análise por espectrometria de massa, pela análise de perfil de proteínas ribossomais (Maldi-Tof MS) e sequenciamento de DNA. As análises químicas foram determinadas por cromatografia líquida de alta eficiência (HPLC). Foram identificadas 20 espécies de bactérias mesófilas, 7 espécies de BAL e 14 espécies de leveduras. Algumas espécies, como *Lactobacillus brevis, L. paracasei, Pantoea Agglomerans, Staphylococcus warneri, Candida parapsilosis, C. orthopsilosis, Cryptococcus flavescen, Bacillus simplex* e *B. thuringiensis* prevaleceram sobre outras. Os açúcares glicose e manitol foram os principais açúcares presentes na azeitona de mesa. Os ácidos acético, cítrico e láctico foram os ácidos detectados em concentrações mais elevadas. Dos microrganismos isolados, foram testadas 14 BAL e 18 leveduras em relação ao potencial probiótico. Seis BAL e seis leveduras mostraram propriedades potenciais para uso como probióticos: as bactérias *Lactobacillus pentosus* CCMA 1768; *L. paracasei* CCMA 1771; *L. paracasei* CCMA 1774; *L. paracasei* CCMA 1770; *L. brevis* CCMA 1766, *L. brevis* CCMA 1762, e as leveduras *Saccharomyces cerevisiae* CCMA1746, *Pichia guilliermondii* CCMA1753, *Candida orthopsilosis* CCMA1748, *C. tropicalis* CCMA1751, *Meyerozyma caribbica* CCMA1758 e *Debaryomyces hansenii* CCMA1761. Esses microrganismos demonstraram capacidade de suportar condições como as encontradas no trato gastrointestinal (pH baixo, sais biliares e temperatura de 37 ° C), e exibiram atividade antimicrobiana contra patógenos. Com relação às propriedades da superfície, os isolados apresentaram capacidade de autoagregação, coagregação com o patógeno *Escherichia coli* e *Salmonella* Enteritidis, adesão às células Caco-2 e HT-29. A metodologia polifásica foi realizada com eficiência para identificar os microrganismos neste trabalho e a análise química ajudou a caracterizar as azeitonas e o processo fermentativo. Esses achados são relevantes, caracterizando azeitonas brasileiras inexploradas anteriormente. O conhecimento da microbiota nativa presente nos frutos dessas azeitonas e das espécies envolvidas na fermentação e sua evolução ao longo do processo pode ser útil para melhorar a qualidade das propriedades sensoriais e preservação deste produto. Além disso, a caracterização da microbiota pode resultar no isolamento de possíveis microrganismos biotecnologicamente importantes. Com os testes preliminares *in vitro*, temos a indicação da segurança e a funcionalidade de bactérias do ácido lático e leveduras isoladas de azeitonas de mesa fermentadas brasileiras como potenciais candidatos probióticos.

Palavras chaves: Fermentação. *Olea europaea*. *Lactobacillus.* Probióticos.

ABSTRACT

Table olives are a fermented product obtained from the fruits of the olive tree (*Olea europaea* L.), which may contain microorganisms with functional properties. This work evaluated the microbial diversity and physicochemical characteristics of fresh fruits and during the fermentation of Brazilian table olives and selected potentially probiotic microorganisms. Yeasts, mesophilic bacteria and lactic acid bacteria (BAL) were isolated from olives of Grappolo 541 and Ascolano cultivars, protected by the Minas Gerais Agricultural Research Corporation (EPAMIG) and kept in collection at the Maria da Fé Experimental Farm - Minas Gerais- Brazil. The isolates were identified by polyphasic techniques: mass spectrometric analysis, ribosomal protein profile analysis (Maldi-Tof MS) and DNA sequencing. Chemical analyzes were determined by high performance liquid chromatography (HPLC). Twenty species of mesophilic bacteria, seven species of BAL and fourteen species of yeast were identified. Some species, such as *Lactobacillus brevis, L. paracasei, Pantoea Agglomerans, Staphylococcus warneri, Candida parapsilosis, C. orthopsilosis, Cryptococcus flavescen, Bacillus simplex* and *B. thuringiensis,* prevailed over others. Glucose and mannitol sugars were the main sugars present in table olives. Acetic, citric and lactic acids were the acids detected at higher concentrations. Of the isolated microorganisms, 14 BAL and 18 yeasts were tested for probiotic potential. Six BAL and six yeasts showed potential properties for use as probiotics: the bacteria *Lactobacillus pentosus* CCMA 1768; *L. paracasei* CCMA 1771; *L. paracasei* CCMA 1774; *L. paracasei* CCMA 1770; *L. brevis* CCMA 1766, *L. brevis* CCMA 1762, and the yeasts *Saccharomyces cerevisiae* CCMA1746, *Pichia guilliermondii* CCMA1753, *Candida orthopsilosis* CCMA1748, *C. tropicalis* CCMA1751, *Meyerozyma caribbica* CCMA1758 e *Debaryomyces hansenii* CCMA1761. These yeasts demonstrated capacity to support conditions like those found in the gastrointestinal tract (low pH, bile salts and temperature of 37 ° C), and exhibited antimicrobial activity against pathogens. Regarding the surface properties, the isolates showed autoaggregation capacity, coaggregation with *E. coli* and *S.* Enteritidis pathogen, adhesion to Caco-2 and HT-29 cells. The polyphasic methodology was efficiently performed to identify the microorganisms in this work and the chemical analysis helped to characterize the olives and the fermentation process. These findings are relevant, characterizing previously unexplored Brazilian olives. Knowledge of the native microbiota present in the fruits of these olives and the species involved in fermentation and its evolution along the process can be useful to improve the quality of sensory properties and preservation of this product. In addition, microbiota characterization may result in the isolation of possible biotechnologically important microorganisms. Preliminary in vitro tests indicate the safety and functionality of lactic acid bacteria and yeasts isolated from Brazilian fermented table olives as potential probiotic candidates.

Keywords: Fermentation. *Olea europaea. Lactobacillus*. Probiotics

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PRIMEIRA PARTE

1. INTRODUÇÃO

Segundo a Norma Portuguesa NP – 3034 (1987), entende-se por "Azeitonas de mesa" o produto preparado a partir de frutos de variedades apropriadas da espécie *Olea europaea* L., em estado de maturação conveniente, submetidos a tratamentos e operações que assegurem as suas características e boa conservação.

Tal conservação pode ocorrer por processos fermentativos, no qual a azeitona de mesa fermentada possui um aroma característico resultante do equilíbrio entre um conjunto de compostos voláteis como hidrocarbonetos, aldeídos, álcoois (etanol), ésteres, cetonas, e outros compostos. A formação destes compostos é um processo dinâmico desenvolvido ao longo da fermentação pela microbiota presente, composta principalmente por bactérias ácido lácticas e leveduras (SABATINI et al., 2008).

A fermentação das azeitonas pode ser realizada de duas maneiras: pelo processo tradicional, através da fermentação espontânea, ou pela adição de cultura starter (PERES et al., 2012). A fermentação caracteriza-se por ocorrer em 3 fases distintas em que os microrganismos vão sucedendo (FERNÁNDEZ et al., 1997). Numa primeira fase ocorrem as bactérias Gram negativas, frequentemente membros da família Enterobacteriaceae e bactérias Gram positivas. A segunda fase é caracterizada por um crescimento de bactérias ácido lácticas e de leveduras e um decréscimo de bactérias Gram negativas. Na terceira fase, é observado um alto crescimento de *Lactobacillus*, tornando-se o grupo de microrganismos predominante, existe também o crescimento simultâneo de leveduras responsáveis pela produção de compostos que conferem cheiro e sabor desejáveis nas azeitonas (PANAGOU et al., 2006; HUTKINS, 2006).

Como a azeitona é um produto tradicionalmente fermentado, esta pode conter microrganismos com propriedades funcionais a serem pesquisados, existindo um grande interesse nesses tipos de alimentos (AYENI et al., 2011). Alimentos suplementados com culturas com potencial probiótico tem assumido um relevante espaço na área científica e tecnológica, impulsionando investigações no seu desenvolvimento, onde os microrganismos isolados de alimentos fermentados têm demonstrado, em estudos *in vitro*, compatibilidade com a microbiota intestinal humana, além de melhoria na sobrevivência (RIVERA-ESPINOZA; GALLARDO-NAVARRO, 2010).

Probióticos são definidos como microrganismos vivos, que podem desempenhar um papel biológico benéfico, quando administradas em doses adequadas. (HILL, et al. 2014). Esses microrganismos benéficos interagem com as superfícies epiteliais no corpo humano, impedindo adesão e invasão de patógenos, melhorando a integridade e controlando a permeabilidade das barreiras epiteliais (GIORGETT et al., 2015).

Assim a seleção de microrganismo com potencial probiótico nas azeitonas de mesa se torna um desafio já que esse produto de origem vegetal ainda é pouco explorado no que se refere a microbiota potencialmente probiótica.

2. REFERENCIAL TEÓRICO

2.1 Características da oliveira e da azeitona, produção e consumo

A oliveira (*Olea europaea* L.) é uma cultura típica de climas mediterrâneos, caracterizados por invernos suaves, com temperaturas não inferiores a 0 º C, e por verões secos e quentes, que atingem temperaturas superiores a 16ºC. Para o cultivo da oliveira uma precipitação de 400-600 mm já é suficiente para o desenvolvimento da planta (SERAFINI et al., 2007).

As azeitonas de mesa são um vegetal fermentado com uma influência pronunciada na dieta e cultura mediterrânea. Atualmente, a produção mundial ultrapassa 2,5 milhões de toneladas / ano (IOC, 2019).

A produção mundial de azeitonas de mesa em quase 30 safras teve uma evolução constante e regular, multiplicando-se durante esse período em 3.1, passando de uma produção de 950.000 toneladas em 1990/91 para aproximadamente 2.953.500 toneladas em 2018/19. Os dados da produção mundial de azeitonas de mesa e produção dos países membros do Conselho Oleícola Internacional (IOC) no período de 1990/91 a 2017/18 estão dispostos na figura 1.

Os países produtores europeus apresentaram um crescimento total de 4% na produção de azeitonas, sendo que a Espanha sofreu uma redução de 6% na produção de azeitonas enquanto Itália e Portugal obtiveram um aumento de 20% e 18%, respectivamente. Ocorreu também um crescimento individual de outros países tais como Argentina, Egito, Israel, Jordânia, Líbano, Marrocos, Tunísia e Turquia

O consumo mundial de azeitonas de mesa aumentou, juntamente com a produção, nas últimas 30 safras, multiplicando-se por 2,86 e aumentou 186 % durante o período 1990/91 – 2017/18. Alguns países que se destacaram no aumento da produção, aumentaram fortemente o seu consumo, entre eles, o Egito que passa de consumir 11.000 ton em 1990/91 para 450.000 ton em 2017/18. A Argélia passa nesse período de 14.000 ton para 280.000 ton e a Turquia de 110.000 ton para 355.000 tonelada. Além disso, os países da União Europeia como um todo, aumentam o consumo, passando de 346.500 para 585.000 toneladas. (IOC,2018).

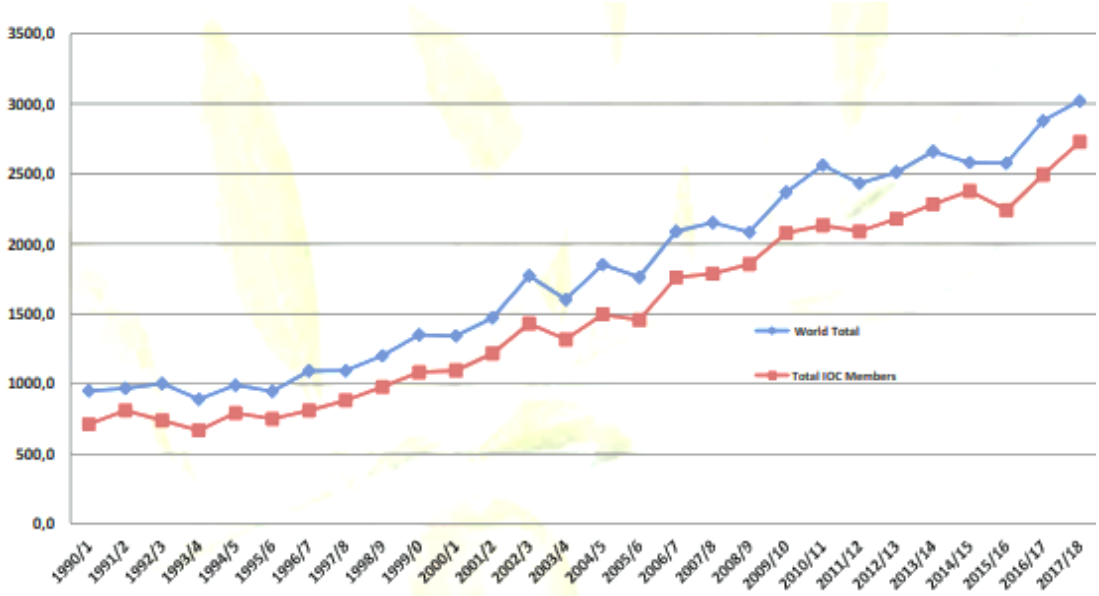

Figura 1 Produção mundial de azeitonas de mesa entre 1990/1 a 2017/18 Fonte: IOC, 2018

A cultura da oliveira se adaptou a diversas regiões da América do Sul. Vários países que possuem condições favoráveis cultivam oliveiras para produção de azeitonas e azeite. Nas Américas, a cultura da oliveira foi introduzida, primeiramente, no México, nos Estados Unidos e no Peru, difundindo-se a partir daí para o Chile e a Argentina (OLIVEIRA et al., 2012).

Diversos são os desafios para conseguir a adaptabilidade da oliveira no Brasil, uma vez que a cultura não é tradicionalmente produzida no país. Estes desafios englobam a escolha da melhor variedade, plantio e manejo dos olivais. A temperatura é o fator ambiental mais importante e que limita a área de produção das oliveiras em condições subtropicais. A planta de oliveira requer um período de inverno com temperaturas entre 5° e 7° C, e alternância de temperaturas entre o dia e a noite que varia entre 4° a 18° C para a transformação de gemas vegetativas em gemas florais. No Brasil, tais condições ocorrem em microclimas como áreas

montanhosas, com altitudes acima de 1000 m e/ou áreas mais ao sul do país com clima subtropical temperado. (TERAMOTO, 2013).

Assim, com a identificação das características de solos e climas regionais, que são fatores importantes para determinar as regiões aptas ao cultivo da oliveira no Brasil, particularmente relacionados a presença de baixas temperaturas, foram realizados plantios comerciais nas regiões Sudeste e Sul do país, tendo destaque os estados de Minas Gerais e Rio Grande do Sul, predominando a variedade "Arbequina", com 50% do plantio, seguido da variedade "Grappolo", com 20 % do plantio, e o plantio da variedade Maria da Fé abrange cerca de 10% a 20% do plantio são de outras variedades como Arbosana, Koroneiki e Ascolano, onde se contabilizam uma área de aproximadamente de 505 ha. (OLIVEIRA et al. 2012).

De acordo com a Associação dos Olivicultores dos Contrafortes da Mantiqueira, MG, que conta com 60 associados, o plantio de oliveira na Região Sudeste está difundido em 40 municípios, com cerca de 400 ha, abrangendo o estado de Minas Gerais e São Paulo. Minas Gerais é referência nacional no estudo da oliveira, não existindo nenhum outro estado no Brasil com a diversidade de genótipos que são encontrados na Fazenda Experimental de Maria da Fé da EPAMIG- Empresa de Pesquisa Agropecuária de Minas Gerais (OLIVEIRA et al. 2012).

As importações de azeitonas de mesa nos primeiros seis meses do ano de 2017/18 safra (setembro de 2017-fevereiro de 2018) mostraram aumento de 5% no Canadá e queda em outros mercados tais como Austrália, Brasil e Estados Unidos, diminuindo 8%, 5% e 4%, respectivamente, no total de importações em comparação com o mesmo período do ano de colheita anterior. O Brasil é o segundo maior importador de azeitonas, e o consumo per capita do país é de 0,5kg $hab.^{-1}$. Mesmo em face do crescimento observado no consumo de azeites e azeitonas, os índices brasileiros encontram-se ainda bem distantes daqueles observados em outros países, sobretudo os da Europa e mediterrâneo. A figura 2 representa as importações brasileiras no período de Março/2019 a Junho/2019, de acordo com a IOOC (2019).

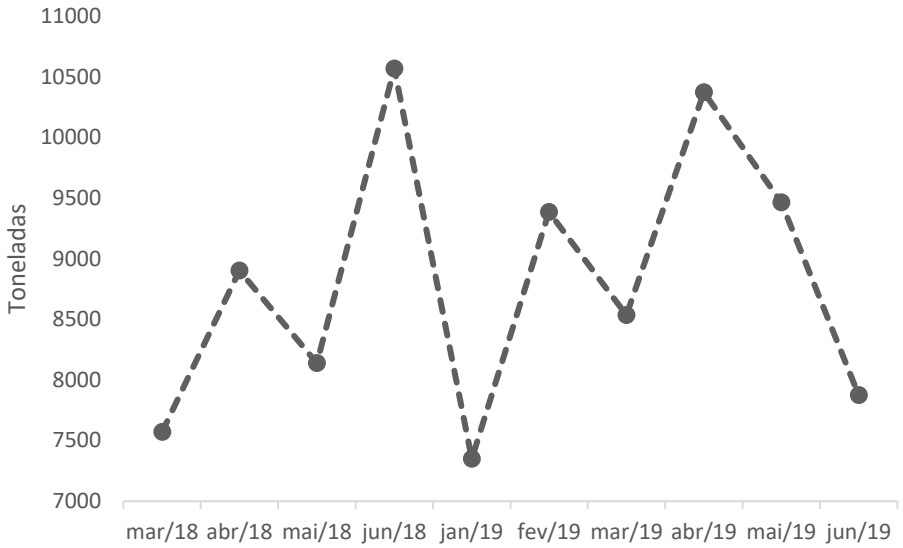

Figura 2 Importação de azeitonas Março de 2018- junho de 2019 Fonte: IOOC (2019)

Os principais fornecedores de azeitonas para o Brasil são os países Argentina, Espanha, Portugal, Peru, e o Chile, sendo a Argentina é o principal fornecedor, correspondendo a 79,5% das importações. A proximidade dos países e os aspectos tarifários, principalmente com advento do MERCOSUL, contribuíram para o fato (MDIC, 2014).

A azeitona consta na lista dos produtos que compuseram o valor da produção agropecuária do Brasil desde 2011, passando a ter sua produção computada nos levantamentos do IBGE. Os últimos dados divulgados estão dispostos na Tabela 1.

Tabela 1 Área plantada ou destinada à colheita, área colhida, quantidade produzida, rendimento médio, valor da produção de Azeitonas - Brasil -2016,2017 e 2018.

Dados Azeitonas Brasileiras	2016	2017	2018
Área (ha) Plantada/Colhida	575/574	963/946	1122/1116
Quantidade produzida (t)	647	1250	1620
Rendimento médio (kg/ha)	1 127	1321	1452
Valor da produção (1 000 R$)	1 636	2779	3521

Fonte: Adaptada de IBGE, Diretoria de Pesquisas, Coordenação de Agropecuária, Produção Agrícola Municipal 2016,2017,2018. (1) A área plantada refere-se à área destinada à colheita no ano. (2) Quantidade produzida em 1 000 frutos e rendimento médio em frutos por hectare.

2.2 A azeitona de mesa

A azeitona (*Olea europaea* L.) é uma drupa ovalada de cor verde que passa a violácea ou preta quando atinge o amadurecimento. Pesa entre 1,5 e 12 g onde a polpa constitui cerca de 70 a 88% do fruto. A polpa da azeitona é maioritariamente constituída por água, de 70 a 75%, e uma fração de lipídeos variando entre 12 a 30%, dependendo do grau de maturação das azeitonas e da variedade (BIANCHI, 2003; PINHEIRO et al., 2005). Apresenta um teor relativamente baixo em açúcares (2-5%), prevalecendo a glucose (1 a 3% do peso da polpa) e a frutose (0,1 a 1,1%) comparativamente à sacarose e ao manitol, e em proteínas (3%), sendo o restante constituído por fibras e minerais (Fernández et al., 1997; Bianchi, 2003; Conde et al., 2008; Sakouhi et al., 2008).

Entende-se por "Azeitonas de Mesa", o produto preparado a partir de frutos, em estado de maturação conveniente, que são escolhidos para a produção de azeitonas cujo volume, relação polpa/caroço, características da polpa, firmeza e facilidade de desprendimento do caroço as tornam adequadas para o processamento, submetidos a tratamentos e operações que assegurem as suas características e boa conservação de modo a remover o seu amargor e a torna-los edíveis. Estas são preservadas por fermentação natural ou por tratamento térmico, com ou sem adição de conservantes e embalados, com ou sem um meio líquido (NORMA PORTUGUESA -3034, 1987).

Tem-se que as azeitonas de mesa são frutos de variedades cultivadas, sadios no estado de maturação e qualidade adequados tal que, são submetidos a procedimentos de elaboração, originando um produto de consumo e de boa conservação. Estas preparações podem, eventualmente, incluir a adição de diversos produtos ou aromatizantes (INSTITUTO DE LA GRASA, 2014).

De acordo com o Codex Alimentarius (2010) e a NP- 3034 (1987), as azeitonas de mesa, assim como todos os produtos de origem alimentar, devem estar isentas de microrganismos patogênicos, bem como de suas toxinas, ou de qualquer outro agente contaminante, podendo, no caso de azeitonas fermentadas e conservadas, encontrar no produto microrganismos que estão envolvidos no processo fermentativo, nomeadamente as *Enterabacteriaceae*, bactérias ácido-lácticas e leveduras (ARROYO-LÓPEZ et al., 2010; BAUTISTA-GALLEGO et al., 2011; PANAGOU et al., 2008).

A azeitona de mesa pode ser classificada de acordo com o grau de maturação, o processamento tecnológico e a forma de apresentação. O Conselho Oleícola Internacional

(IOC, 2004), entidade que regula o setor dos azeites e da azeitona de mesa a nível internacional, considera três tipos de azeitona de mesa, de acordo com seu grau de maturação (Tabela 2).

Tabela 2 Classificação da azeitona de mesa quanto ao grau de maturação

Tipo de azeitona	Classificação
Verde	Colhida durante o período de amadurecimento e que tenha atingido o tamanho normal (aproximadamente 3g a 5g). A cor do fruto pode variar de verde a amarelo-palha;
Mista	Obtida a partir de frutos colhidos antes de atingirem a completa maturação, com uma cor variável entre tons rosados e acastanhados;
Preta	Colhida completamente madura ou ligeiramente antes. A cor do fruto pode variar de avermelhado-escuro a castanho-escuro, passando por tons violáceos.

Fonte (IOC, 2004)

A conservação de azeitonas de mesa pode ser realizada em salmoura, em sal, em atmosfera modificada, através de processos térmicos, com conservantes ou ainda através da adição de agentes acidificantes (IOC, 2004). Em consideração aos diferentes processos tecnológicos, a azeitona pode ter diferentes designações, entre as quais: azeitona curada em salmoura, azeitona ao natural em salmoura, azeitona escurecida por oxidação e azeitona desidratada e/ou enrugada (Tabela 3).

Tabela 3 Classificação da azeitona de mesa relativamente ao processo tecnológico

Tipo de azeitona	Classificação
Azeitona curada em salmoura	É submetida a um tratamento alcalino, embalada em salmoura, onde ocorre fermentação parcial ou completa, e conservada ou não por agentes acidificantes;
Azeitona ao natural em salmoura	Diretamente colocada em salmoura, onde ocorre fermentação parcial ou completa, e conservada ou não por agentes acidificantes;
Azeitona escurecida por oxidação	Obtida a partir de frutos que não atingiram a maturação completa e escurecida mediante oxidação após o tratamento alcalino. Deve ser acondicionada em salmoura e preservada por esterilização. A sua cor preta é homogênea;
Azeitona desidratada e/ou enrugada	É submetida ou não a um tratamento alcalino, conservada em salmoura ou parcialmente desidratada em sal seco e/ou por aquecimento ou por qualquer outro processo tecnológico.

Fonte (IOC, 2004)

As azeitonas em salmoura são um dos tipos de fabricação mais utilizados comercialmente, esta preparação em salmoura é de grande importância para a qualidade do produto final, pois a concentração de sal contribui para a redução da atividade da água retardando ou interrompendo a atividade de microrganismos indesejáveis que possam causar deterioração nas azeitonas. Tem-se que a salmoura estimula a ação de microrganismo que são responsáveis pelo processo fermentativo e também pela redução do sabor amargo, causado pelo composto fenólico oleuropeína. Além disso a salmoura contribui para a redução do pH, aumento da força iónica da solução, difusão de componentes solúveis e redução a solubilidade do oxigênio na água, assegurando assim a segurança do produto final durante o período de armazenamento (BAUTISTA-GALLEGO et al., 2011; COI, 2014; PANAGOU et al., 2011).

O aroma característico das azeitonas de mesa fermentadas resulta do equilíbrio entre um conjunto de compostos voláteis como hidrocarbonetos, álcoois (etanol), cetonas, aldeídos, ésteres entre outros compostos. A formação destes compostos é um processo dinâmico desenvolvido ao longo da fermentação pela microbiota presente principalmente composta por bactérias ácido lácticas e leveduras (SABATINI et al., 2008). O etanol é responsável principalmente pelo odor característico do produto final, entre outras propriedades organolépticas, sendo um dos produtos finais do metabolismo das leveduras podendo também ser produzido por BAL heterofermentativas (TASSOU et al., 2002).

No final do processo de fermentação, as azeitonas submetidas a uma fermentação natural, devem apresentar uma percentagem mínima de 6% de NaCl, um pH máximo de 4,3 e uma percentagem de acidez de 0,3 % ácido láctico (p/v), para garantir uma boa conservação e propriedades características do produto. As azeitonas e a salmoura devem estar isentas de qualquer tipo de deterioração microbiana, bem como estar livres de microrganismos patogênicos e metabolitos produzidos pelos mesmos. O volume de produto coberto com salmoura não deve nunca ocupar menos de 90% do volume total do recipiente de embalagem (IOC, 2004).

2.3 Produção industrial de azeitonas

Nas indústrias de azeitonas, as azeitonas são colhidas nos olivais e transportadas para a indústria. Após a entrada na indústria, as azeitonas sofrem um tratamento alcalino, também conhecido como adoçamento, com uma solução de hidróxido de sódio (NaOH) de concentração 1,5%-3,5%, num período de tempo de 6-10 horas. Esta operação tem como objetivo remover o sabor amargo, causado pela oleuropeína, e os açúcares, transformando-os em ácidos orgânicos que favorecem a fermentação láctica e aumentam a permeabilidade do fruto (IOC, 2014). Esta etapa também é fundamental para eliminação de alguns compostos fenólicos que são tóxicos para microrganismos e fornecer ao meio nutrientes necessários para o crescimento dos microrganismos durante o processo fermentativo.

As azeitonas são mantidas submersas nesta solução até o NaOH penetrar 2 /3 da distância da epiderme ao caroço, onde o NaOH demora aproximadamente de uma a duas horas a penetrar na epiderme da azeitona. A temperatura ótima para que ocorra está penetração é de 30^{o}C. Em seguida, as azeitonas sofrem duas ou três lavagens, com o objetivo de remover o excesso de NaOH (IOC, 2014; INSTITUTO DE LA GRASA, 2014). A primeira

lavagem dura aproximadamente duas a três horas, onde o hidróxido de sódio continua a penetrar na azeitona, já a segunda lavagem dura entre 10 e 12 horas, também se aplica a opção de lavagem única durando aproximadamente entre 12 e 14 horas.

Posteriormente, as azeitonas são imersas em salmoura com concentração inicial de 10-12% (p/v) de cloreto de sódio (NaCl). Logo após a colocação em salmoura, é recomendado esperar aproximadamente uma hora para atingir um equilíbrio osmótico, a fim de que a salmoura se transforme em um meio de cultura propício ao desenvolvimento de microrganismos responsáveis pela fermentação das azeitonas, em seguida inicia-se o processo fermentativo das azeitonas. Como consequência do crescimento microbiano, os substratos presentes nos frutos se transformam em diferentes produtos metabólicos, os quais constituem o processo fermentativo das azeitonas. Em processos industriais é requerida a reposição periódica da salmoura para manter sempre as azeitonas cobertas, evitando assim o escurecimento dos frutos e aumentando a concentração salina.(OLIVEIRA, et. al. 2012).

Uma fermentação adequada ocorre no mínimo em 90 dias. Após esse período, as azeitonas são lavadas, selecionadas e calibradas. A salmoura é substituída e as azeitonas podem ser embaladas inteiras, descaroçadas, com recheio, entre outras formas. Antes de serem embaladas as azeitonas devem ser agrupadas de acordo com a cor, textura e sabor. Muitas indústrias empregam o processo de pasteurização a 80ºC para melhor conservação do produto. A salmoura utilizada no embalamento deve ter uma acidez em torno de 0,4 a 0,7%, e uma concentração de NaCl de aproximadamente 0,5 a 3,5% e nesta salmoura pode-se ou não adicionar conservantes como ácido ascórbico ou benzoico ou os seus sais de sódio e potássio. (OLIVEIRA, et. al. 2012).

2.4 Métodos de processamento de azeitonas de mesa

O processamento da azeitona tem como principal objetivo a remoção do sabor amargo característico dos frutos por hidrólise/difusão dos compostos fenólicos, especialmente o composto oleuropeína (APONTE et al., 2010). Independentemente do tipo de processamento adotado, este deve ser realizado respeitando o código de boas práticas sanitárias, de modo a garantir características químicas e microbiológicas do produto (DABBOU et al., 2012).

Considerando os vários tratamentos que podem ser aplicados nas azeitonas de mesa, os que apresentam maior importância a nível mundial são o Sevilhano ou Espanhol (azeitonas verdes), o Californiano ou Americano (azeitonas pretas oxidadas) e o Grego (de fermentação

natural) (BAUTISTA-GALLEGO et al., 2011; DABBOU et al., 2012; GHANBARI et al., 2012; TOFALO et al., 2012a).

Nas azeitonas processadas através do método Espanhol (azeitonas verdes) a fermentação é conduzida majoritariamente por BAL enquanto nas azeitonas processadas através do método Grego (fermentação espontânea) os microrganismos responsáveis pela fermentação são leveduras e bactérias ácido lácticas, que constituem uma fração da microbiota total (CAMPANIELLO et al., 2005).

Para a preparação de azeitonas de fermentação natural espontânea, os frutos colhidos são lavados com água para remover a sujidade superficial e colocadas em salmoura com uma concentração de sal que pode variar de 6% a 10% (NYCHAS et al., 2002). Em seguida, inicia-se o processo de fermentação "espontânea", conduzida majoritariamente por leveduras, bactérias gram-negativas e bactérias ácido-lácticas (APONTE et al., 2010, NOUT, ROMBOUTS, 2000; OLIVEIRA et al., 2004; PIGA et al., 2005; PANAGOU et al., 2011). Esta microbiota é determinada pelo substrato disponível para o crescimento dos microrganismos, pela concentração de sal e também pela temperatura, pH, condições de anaerobiose ou aerobiose, e presença de compostos antimicrobianos, como por exemplo os compostos fenólicos (NYCHAS et al., 2002; PANAGOU et al., 2008; TOFALO et al., 2012b).

A fermentação espontânea é um processo moroso, essencialmente devido à lenta difusão dos açúcares e de outros compostos solúveis através da epiderme da azeitona para a salmoura, e devido à presença do composto oleuropeína e outros compostos fenólicos que apresentam atividade antimicrobiana, sendo o equilíbrio alcançado depois de alguns meses de fermentação. As leveduras, que estão envolvidas no processo de fermentação de azeitonas de mesa, produzem compostos voláteis e metabolitos com atributos organolépticos importantes que determinam a qualidade e o sabor do produto final (HERNÁNDEZ et. al., 2007; ARROYO-LÓPEZ et al., 2008). Algumas espécies estão associadas à hidrólise de oleuropeína (composto fenólico responsável pelo amargor característico dos frutos frescos da azeitona), catalisada pela enzima glicosidase, contribuindo para a remoção do amargor que está presente naturalmente nas azeitonas. Entretanto, algumas estirpes de microrganismos podem ser deteriorantes no processo de fermentação das azeitonas causando formação de gases, turvação da salmoura, amolecimento da polpa, produção de sabores e odores indesejáveis ou abaulamento das embalagens (ARROYO-LÓPEZ et al., 2008).

Alguns gêneros de leveduras que podem ser encontradas na fermentação de azeitonas são *Pichia*, *Candida*, *Kluyveromyces*, *Cryptococcus*, *Saccharomyces*, *Trichosporum*, *Debaryomyces*, *Rhodoturula*, *Torulaspora* (HERNANDÉZ et al., 2007). As espécies *Pichia anomala, Pichia membranaefaciens, Candida boidinii, Debaryomyces hansenii, Rhodothorula glutinis* e *Saccharomyces cerevisiae* usualmente já foram encontradas em diversos processos de fermentação de azeitonas (ARROYO-LOPEZ et al., 2008b; RODRIGUEZ-GOMEZ et al., 2010).

Ao final do processo fermentativo, as azeitonas fermentadas naturalmente apresentam um sabor mais acentuado com um ligeiro amargor, devido especificamente à presença de polifenóis residuais, que resulta em azeitonas com alto valor nutritivo, textura e cor característica (BARRANCO et al., 2001; ROMERO et al., 2004; KANAVOURAS et al., 2005; CABEZAS, 2011; PANAGOU et al., 2011).

Nas azeitonas verdes, também denominadas azeitonas verdes curadas em salmoura, azeitonas verdes estilo Sevilhano ou Espanhol, as azeitonas são colhidas quando atingem uma cor verde-amarelada, e submetidas a um tratamento anaeróbio com NaOH durante algumas horas. A solução alcalina é retirada e as azeitonas são lavadas repetidamente com água, que remove parte da oleuropeína e os seus produtos de hidrólise, outros polifenóis e alguns dos açúcares fermentáveis (FERNÁNDEZ et al., 1997). Em seguida, os frutos são colocados em salmoura, contendo 7% a 10% de NaCl, para que ocorra o processo fermentativo (NOUT, ROMBOUTS, 2000). Uma vez fermentadas, as azeitonas são selecionadas e classificadas por tamanhos para serem embaladas, podendo ou não ocorrer uma pasteurização.

No processamento das Azeitonas pretas oxidadas, também conhecido por Estilo Californiano ou Americano, as azeitonas utilizadas são colhidas, antes de atingir sua plena maturação (REJANO et al., 2010). As azeitonas pretas são submetidas à tratamento aeróbico com NaOH para eliminação dos compostos amargos e uniformização da cor. Antes do processamento as azeitonas podem, se necessário, ser conservadas vários meses sob condições anaeróbias numa salmoura, assim são imersas em uma solução contendo NaCl 8-10% (p/v) sem pré-tratamento com solução de NaOH, ocorrendo então uma fermentação natural que envolve uma microbiota mista de bactérias Gram negativas, bactérias ácido lácticas e leveduras. Após um período inicial de fermentação vigorosa, bactérias ácido lácticas e leveduras coexistem até ao fim do processo (NISIOTOU et al., 2009, NYCHAS et al., 2002; PANAGOU et al., 2008).

No processamento no Estilo Californiano ou Americano as azeitonas são sujeitas a tratamentos sucessivos com soluções de NaOH, por diferentes períodos de tempo, para que ocorra uma penetração progressiva desde a pele à polpa e, por fim, ao caroço. No fim de cada tratamento alcalino as azeitonas são lavadas com água, juntamente com injeção de ar sob pressão. Este tratamento alcalino aeróbio permite o escurecimento progressivo da pele e da polpa do fruto. Depois da obtenção da cor almejada, as azeitonas pretas ainda são lavadas e arejadas até atingir aproximadamente um pH 8 (MARSILIO et al., 2001). A coloração negra obtida nas azeitonas é instável e pode perder-se ao longo da vida útil do produto acabado, assim na última água utilizada para a lavagem final das azeitonas, são adicionados 0,1% de gluconato ferroso para estabilizar a cor alcançada e desejável na oxidação (FERNÁNDEZ et al., 1997).

O processo de fermentação de azeitonas de modo geral varia entre 3 a 4 semanas, mas também pode levar mais de um ano, dependendo dos fatores intrínsecos pH, atividade de água, difusão de nutrientes da polpa, e dos extrínsecos como temperatura, disponibilidade de oxigênio, concentração de NaCl e do número de microrganismos presentes (LUH, WOODROOF,1988; HURTADO et al., 2008).

2.5 Probióticos

Atualmente, os consumidores estão se tornando mais conscientes da saúde e preocupados com o valor benéfico dos alimentos, direcionando assim os fabricantes a enfatizar a promoção de alimentos funcionais. Portanto, a chave para o sucesso do marketing e a aceitação de novos alimentos depende não apenas do conceito de qualidade dos alimentos em toda a cadeia, mas também das funcionalidades dos alimentos com valor agregado (KHEDKAR, et al. 2017). O desenvolvimento de formulações probióticas de alimentos é uma área de pesquisa essencial para o futuro mercado funcional de alimentos. As previsões econômicas esperam um aumento de 3,3 para 7 bilhões de dólares para o mercado global de suplementos alimentares probióticos de 2015 a 2025 (STATISTA, 2019).

Segundo a Associação Científica Internacional para Probióticos e Prebióticos mantida em 2014, a definição apropriada do termo probiótico consiste “Microrganismos vivos que, quando administrados em quantidade adequadas, conferem um benefício à saúde ao hospedeiro” (HILL, et al. 2014). Diversas evidências indicam que os efeitos destes probióticos no hospedeiro são específicos da cepa, isto é provavelmente devido aos diferentes

mecanismos de ação e produção de diferentes metabólitos produzidos por cada microrganismo (TORRES, et al. 2019).

Diretrizes e informações da Organização das Nações Unidas para a Alimentação e Agricultura (FAO) / Organização Mundial da Saúde (OMS) demonstram a necessidade de as cepas probióticas permanecerem intactas no trato intestinal para garantir a saúde promover efeitos ao entrar no local de ação, independentemente do modo de entrega aplicado. Por exemplo, para garantir isso, foi afirmado que o chamado nível "terapêutico mínimo" de probióticos viáveis os microrganismos devem ter pelo menos 10^6 UFC / g de células viáveis durante o prazo de validade do produto (NEFFE-SKOCINSKA, et al. 2018).

Tradicionalmente, existem muitas espécies diferentes de probióticos amplamente utilizados. A levedura *Saccharomyces cerevisiae* (boulardii) é a cepa mais utilizada. Outros probióticos bacterianos compreendem principalmente espécies de *Lactobacillus* e *Bifidobacterium*. Estes incluem L. *rhamnosus, L. plantarum, L. sporogens, L. reuteri, L. casei, L. bulgaricus, L. delbrueckii, L. salivarius, L. johnsonii,* e *L. acidophilus*. Além disso, *B. bifidum, B. bifidus, B. lactis, B. longum, B. breve* (Yakult), e *B. infantis* também são comumente usados. Outros probióticos comercialmente disponíveis incluem *Streptococcus thermophilus, Streptococcus acidophilus, Lactococcus lactis, Enterococcus* SF68 e *Escherichia coli Nissle* 1917 (sorotipo O6: K5: H1) (FIJAN, 2017).

A microbiota intestinal normal desempenha um papel importante na manutenção da saúde humana. Cepas de bactérias ou leveduras com o potencial probiótico comprovado auxiliam na manutenção da homeostase intestinal através de uma ampla gama de funções. Devido ao avanço das ferramentas de pesquisa, mais cepas com potencial probiótico podem ser caracterizadas e a suplementação a dieta com essas bactérias, ou leveduras tem melhorado digestão e saúde dos seres humanos (KUMAR, et al.,2019).

A maioria dos microrganismos probióticos comercializados são originalmente isolados de amostras de fezes humanas, de forma a maximizar a probabilidade de compatibilidade com a microbiota intestinal e melhorar suas chances de sobreviver a esse ambiente. Entretanto, microrganismos isolados de alimentos fermentados têm demonstrado habilidades probióticas em estudos "*in vitro*", portanto, os alimentos fermentados tradicionais constituem reservatório para pesquisas por novas cepas com novas propriedades funcionais (RIVERA-ESPINOZA; GALLARDO-NAVARRO, 2010, AYENI et al., 2011).

Estudos de ecologia microbiana de processos fermentativos de azeitona evidenciam uma sucessão de populações mistas (*Enterobacteriaceae*, bactérias lácticas e leveduras), bem

como uma fonte promissora de novas estirpes de bactérias lácticas com aptidão probiótica (LAVERMICOCCA et al., 2002; HALLER et al. 2001; MORAD, NOUREDDINE, 2006; BAUTISTA-GALLEGO et al., 2013; ARGYRI et al., 2013).

2.6 Potencial probiótico de microrganismos isolados de azeitonas

Alguns estudos já foram realizados para avaliar a potencialidade probiótica de microrganismos isolados da fermentação de azeitonas de mesa. No trabalho de Moreira (2013) foi realizado um estudo *in vitro* das propriedades probióticas das bactérias lácticas *Lactobacillus pentosus* B96, *Lactobacillus plantarum* 614 e *Lactobacillus paraplantarum* A1 isoladas de azeitonas ao natural. Foi verificado que estas cepas apresentam potencial probiótico, e estas foram adicionadas às salmouras de fermentação (escala piloto), atuando como culturas adjuntas, e os resultados sugerem que possuem propriedades tecnológicas promissoras, nas quais a adição das cepas de *L. pentosus* B96 e *L. paraplantarum* A1 mantiveram boas características organolépticas no produto.

Nesse mesmo estudo, foi analisado a viabilidade das bactérias com potencial probiótico nas azeitonas fermentadas naturalmente durante 90 dias, ao fim desse período o produto registrou a quantidade mínima de bactérias lácticas exigida pela FAO/WHO para um alimento probiótico (10^6 UFC/g) quando armazenado a 22 ºC, assim este estudo sugeriu que a azeitona ao natural, pode constituir um veículo biológico eficiente para ingestão de probióticos, quando embalada em salmoura e conservada à temperatura ambiente.

As bactérias do ácido láctico *Leuconostoc mesenteroides*, *Leuconostoc Pseudomesenteroides*, *Lactobacillus plantarum*, *Lactobacillus pentosus*, *Lactobacillus paraplantarum* e *Lactobacillus paracasei subsp. Paracasei* isoladas de azeitonas de mesa foram testadas em relação ao seu potencial probiótico no trabalho de Argyri et al. (2013). As espécies *L. pentosus, L. plantarum e L. paracasei subsp. paracasei* tiveram suas propriedades probióticas *in vitro* desejáveis semelhantes ou até melhores que as estirpes probióticas de referência utilizadas neste estudo (*L. casei Shirota* e *L. rhamnosus*).

No estudo realizado por Blana et al. (2014) o desempenho de duas cepas de bactérias do ácido láctico, nomeadamente *Lactobacillus pentosus* B281 e *Lactobacillus plantarum* B282, previamente isolado de azeitonas de mesa fermentadas industrialmente e com potencial probiótico *in vitro* comprovado, foram investigadas como culturas iniciadoras em fermentação de estilo espanhol de azeitonas verdes da cv. Halkidiki (Grécia). A fermentação foi realizada à

temperatura ambiente em duas concentrações iniciais de sal diferentes (8% e 10%, p / v de NaCl) nas salmouras. As cepas foram inoculadas isoladas e como culturas combinadas e a dinâmica populacional na superfície das azeitonas foi monitorada. Ambas as cepas probióticas colonizaram com sucesso a superfície da azeitona em populações variando de 6,0 a 7,0 log UFC / g ao longo da fermentação. A bactéria *L. pentosus* B281 apresentou maior contagem em ambos os níveis de sal no final do processo fermentativo e foi dominante nas fermentações onde as duas cepas foram utilizadas como culturas combinadas.

Bautista-Gallego et al. (2013) estudaram as potenciais propriedades probióticas de bactérias do gênero *Lactobacillus* associados a azeitonas de mesa. Foram identificadas as bactérias *Lactobacillus pentosus, Lactobacillus plantarum* e *Lactobacillus paraplantarum*. A maioria apresentou alta capacidade de autoagregação, baixa hidrofobicidade e menor sobrevivência para a digestão gástrica do que para a pancreática. A bactéria *L. pentosus* foi caracterizada com um potencial promissor probiótico.

Rodríguez-Gómez et al. (2013) utilizaram quatro cepas de *L. pentosus* isoladas de azeitonas de mesa da cultivar Manzanilla (Espanha) fermentadas pelo processamento estilo espanhol como culturas iniciadoras em azeitonas. Estas bactérias foram selecionadas de acordo com diversos testes fenotípicos *in vitro* relacionados ao potencial probiótico, como resistência à digestão gástrica e pancreática, auto-agregação, hidrofobicidade, produção de bacteriocinas, atividade hemolítica e capacidade de desconjugar o sal biliar e utilizados no presente estudo como inóculos. O experimento consistiu em um tratamento com fermentação espontânea sem adição de microrganismos, e outros tratamentos com adição das bactérias *L. pentosus.* Quando comparado com o processo espontâneo, o uso desses microrganismos como iniciador levou a maiores populações de bactérias de ácido lático do que as populações de leveduras, e uma diminuição nos níveis de Enterobacteriaceae e uma rápida acidificação das salmouras. Diversos perfis genéticos entre as populações de bactérias do ácido láctico foram encontrados no final da fermentação.

Guantario et al. 2018, caracterizaram a nível de espécie de 49 cepas de bactérias do ácido lático (LAB) derivadas de azeitonas de mesa Nocellara del Belice fermentadas com os métodos espanhol ou castelvetrano e cepas de *L. pentosus* e *L. coryniformis* foram submetidas a triagem adicional para avaliar seu potencial probiótico, utilizando uma combinação de abordagens *in vitro* e *in vivo*. O estudo revelou que uma linhagem de *L. pentosus* e uma de *L. coryniformis* induziu significativamente efeitos de longevidade e proteção contra infecções mediadas por patógenos em testes *in vivo*. Além disso, ambas as cepas exibiram adesão às

células epiteliais intestinais humanas Caco-2. No geral, os autores concluíram que esses resultados sugerem características benéficas para novas cepas de LAB, o que os torna candidatos promissores como iniciantes para a fabricação de azeitonas de mesa fermentadas com valor agregado probiótico.

No trabalho recente de Benítez-Cabello et al. 2019, isolados de BAL da superfície das oliveiras das cultivares Manzanilla, Gordal e Aloreña, processadas como azeitonas verdes ao estilo espanhol ou salgadas diretamente (naturais) foram estudados. *L. pentosus* Lp13 (ou LPG1) foi o genótipo mais frequentemente encontrado durante fermentação e apresentou um bom desempenho para muitos dos aspectos tecnológicos probióticos, apresentando potenciais características probióticas (resistência ao pH, auto e coagregação com espécies de leveduras, sobrevivência a digestão pancreática, ausência de resistência a antibióticos, inibição de patógenos, presença de genes *bsh*, remoção de colesterol, etc.)

Uma coleção de 31 linhagens de *Lactobacillus pentosus* isoladas de azeitonas verdes de mesa Aloreña fermentadas naturalmente foi avaliada quanto ao seu potencial probiótico no trabalho de Montoro et al. 2016. Várias linhagens poderiam ser consideradas promissoras probióticas, pois apresentavam boa capacidade de crescimento e sobrevivência em condições gastrointestinais simuladas (pH ácido de 1,5, até 4% dos sais biliares e 5 mM de nitrato), boa capacidade de autoagregação e coagregação com bactérias patogênicas (*Listeria innocua*, *Staphylococcus aureus*, *Escherichia coli* e *Salmonella* Enteritidis). Além disso, as cepas exibiram aderência às linhas celulares intestinais e vaginais, atividade antagônica contra bactérias patogênicas, e capacidade de fermentar vários prebióticos. Os autores selecionaram a cepa *Lactobacillus pentosus* CF2-10N apresentando o melhor perfil probiótico, sendo de grande interesse em estudos futuros, e concluíram que as azeitonas de mesa Aloreña fermentadas espontaneamente são consideradas uma fonte natural de microrganismos com potencial probiótico, como a espécie *L. pentosus*.

Nos últimos anos, algumas pesquisas têm focalizado atenção para determinar efeitos favoráveis que as leveduras podem proporcionar ao processamento de azeitona de mesa, no trabalho de Bonatsou et al. (2015) foram avaliados o potencial tecnológico (resistência a NaCl e produção de lipase, esterase e β-glucosidase) e características probióticas (atividade de fitase, sobrevivência a digestões gástricas e pancreáticas) de 12 cepas de levedura originalmente isoladas e fermentações de azeitona de mesa preta natural grega. A análise de classificação multivariada realizada revelou que as cepas mais promissoras (claramente discriminadas do resto dos isolados) foram *Pichia guilliermondii* Y16 (que mostrou

globalmente a maior resistência a NaCl e digestões simuladas) e *Wickerhamomyces anomalus* Y18 (com as atividades enzimáticas tecnológicas totais mais altas). Os resultados do trabalho sugerem a avaliação dessas duas leveduras selecionadas como iniciadores multifuncionais, sozinhas ou em combinação com bactérias do ácido láctico, em fermentações de azeitona de mesa.

As propriedades tecnológicas de 15 cepas da levedura *Debaryomyces hansenii* e 32 cepas de *Torulaspora delbrueckii* isoladas de azeitonas pretas fermentadas pelo estilo grego foram analisadas no trabalho de Psani, Kotzekidou (2006). Todas as cepas foram capazes de crescer a 15°C, pH baixo (2,5), e a maioria das cepas conseguiu crescer a 10% (w / v) NaCl, tolerar 0,3% (p/v) de sais biliares e mostraram atividade lipolítica. Apenas 33% das cepas estudadas de *D. hansenii* e 9% das cepas de *T. delbrueckii* podem hidrolisar 1% (p / v) de oleuropeína. Algumas cepas foram capazes de inibir cepas de *L. monocytogenes*, *B. cereus* e *S. typhimurium*. A possível inibição de agentes patogênicos de origem alimentar, bem como o potencial probiótico das cepas, sugeri que as leveduras *D. hansenii* e *T. delbrueckii* podem ser utilizadas como adjuntas iniciais para contribuir com a melhoria da qualidade de azeitonas fermentadas.

Leveduras isoladas da fermentação natural de azeitonas de mesa, especialmente dos gêneros *Kluyveromyces*, *Pichia* e *Candida* (SILVA et al., 2011) das cultivares Galega e Cordovil, têm sido caracterizadas com potencial probiótico através de ensaios independentes de resistência aos sais biliares e pH baixo.

No estudo de Bonatsou et al. 2018, as características probióticas e tecnológicas de 49 linhagens de leveduras, previamente isoladas da fermentação natural de azeitona preta de mesa foram analisadas. Os resultados indicaram que as leveduras apresentaram aumento da tolerância a altas concentrações de sal em diferentes valores de pH comumente encontrados durante o curso da fermentação, indicando sua capacidade de dominar o processo. Além disso, a maioria das cepas apresentou alta capacidade de autoagregação e sobrevivência à digestão gástrica, pancreática e global. A cepa *Aureobasidium pullulans* Y42 mostrou alta capacidade de aderir às células Caco-2.

Porru et al. (2018), isolaram leveduras de salmoura de azeitonas de mesa pretas da variedade Bosana. As espécies dominantes foram *Wickerhamomyces anomalus* e *Nakazawaea molendini-olei*, embora *Candida diddensiae, Candida boidinii, Zygotorulaspora mrakii* e *Saccharomyces cerevisiae* também estavam presentes em proporções mais baixas. Foram realizados testes *in vitro* em relação ao potencial probiótico dessas leveduras, como remoção

do colesterol, digestão gástrica e pancreática, ensaios de biofilmes isoladamente e na cocultura características de *Lactobacillus pentosus*), e os resultados mostraram que *W. anomalus* Wa1 exibiu as melhores características, entretanto, as leveduras *S. cerevisiae* Sc24 e *C. boidinii* Cb60 também apresentaram características probióticas promissoras.

2.7 Efeitos biológicos dos probióticos

Através de suas atividades metabólicas e interações moleculares com células hospedeiras, os microrganismos comensais do intestino são capazes de influenciar a fisiologia do trato gastrointestinal, bem como órgãos e sistemas extra intestinais, sendo determinante na saúde humana (Schroeder, Backhed 2016). Várias pesquisas foram dedicadas ao estudo sobre como a ingestão de microrganismos probióticos na dieta pode modular a composição e atividade no trato gastrointestinal (Park et al. 2013; Park et al. 2017; Falcinelli et al. 2015; Bagarolli, et al. 2017).

No trabalho de Du et al, 2018, foi avaliado os efeitos do probiótico *Bacillus amyloliquefaciens* SC06 (Ba), originalmente isolado do solo, na dieta de leitões como alternativa aos antibióticos (aureomicina), principalmente na barreira epitelial intestinal e na função imunológica. Os resultados demonstraram que o probiótico conseguiu aumentar a barreira das células epiteliais intestinais e a função imunológica, melhorando a estrutura da mucosa intestinal.

Alguns probióticos demonstraram aumentar fagocitose ou atividade natural de células "killer" e interagem diretamente com células dendríticas (KLAENHAMMER, et al. 2012). Alguns também demonstram a capacidade de regular positivamente a secreção de anticorpos melhorando as defesas contra patógenos e aumentando as respostas às vacinas (PRZEMSKA-KOSICKA, et al. 2017; VITETTA, et al. 2017; CHILDS, et al. 2014). Estirpes probióticas podem também aumentar os níveis de citocinas anti-inflamatórias com implicações para diminuir o câncer de cólon e colite (KLAENHAMMER, et al. 2012, ROWLAND, et al., 2018).

Alguns estudos foram realizados para verificar se os probióticos são capazes de apoiar a microbiota intestinal comensal e reduzir a infecções por patógenos. No estudo realizado por Roychowdhury, et al. 2018 foi estudado a bactéria *Clostridium difficile*, cujo a infecção causada por esse microrganismo aumenta a morbidade do paciente, a mortalidade e os custos com saúde. O tratamento com antibióticos induz a disbiose intestinal e é um dos principais

fatores de risco para a colonização da bactéria *C. difficile* e o tratamento da infecção por este microrganismo. Este estudo investigou o microrganismo *Faecalibacterium prausnitzii* e um prebiótico, ambos conhecidos por produzir butirato e ser anti-inflamatório e imunomodulador, na colonização de *C. difficile* e na integridade intestinal de camundongos. A suplementação com essa bactéria e prebiótico pode apoiar respostas imunes inatas e minimizar a carga bacteriana e os efeitos negativos durante a exposição a antibióticos e *C. difficile*.

Estudos clínicos demonstraram que diversas cepas de microrganismos probióticos proporcionam vários benefícios à saúde do hospedeiro, incluindo a síntese de vitaminas e ácidos graxos de cadeia curta. Bactérias com a capacidade de sintetizar tais compostos podem ser usadas como ferramentas para recuperar a ingestão de energia, otimizando a produção de ATP a partir de alimentos ou pela fermentação de certas fibras no trato gastrointestinal (GIT). Bactérias probióticas podem sintetizar ácidos graxos de cadeia curta (acetato, butirato e propionato) e vitaminas do grupo B (riboflavina, folato e tiamina), acredita-se que esses compostos melhorem os distúrbios metabólicos (LEBLANC, et al. 2017).

Existem muitos estudos que sugerem que os probióticos podem ser ingeridos como suplemento dietético para prevenção, intervenção e tratamento de distúrbios metabólicos, incluindo a diabetes (TONUCCI et al. 2017a, b). No geral, para a Diabetes Mellitus tipo 2, uma das doenças mais prevalentes mundialmente, o efeito benéfico dos probióticos pode estar relacionado com a melhoria da integridade intestinal, diminuindo os níveis de lipopolissacarídeos (LPSs), aumento de incretinas, diminuição do estresse do retículo endoplasmático (ER) e subsequente melhora da sensibilidade periférica à insulina (PARK et al. 2015; BALAKUMAR et al. 2018; LIM et al. 2016). Os probióticos também podem exercer efeitos antidiabéticos, melhorando a tolerância à glicose, regulação do metabolismo lipídico, melhorando o status antioxidante e modulando a microbiota intestinal e ácidos graxos de cadeia curta (SIMON et al. 2015; AKBARI et al. 2016). Além disso, os probióticos reduzem a resposta inflamatória, a resposta autoimune ao estresse oxidativo (GOMES et al. 2014; SINGH et al. 2017).

Os efeitos da cepa *Lactobacillus acidophilus* DDS-1, fabricada pela Nebraska Cultures, no alívio sintomático para intolerância à lactose foi estudado no trabalho de Pakdaman, et al. 2016. O estudo concluiu que o microrganismo probiótico *Lactobacillus acidophilus* DDS-1, é segura para consumir e melhora a pontuação dos sintomas abdominais em comparação com o placebo (sem ingestão de probióticos) em relação à diarréia, caimbras e vômitos durante um desafio agudo de intolerância à lactose.

A aterosclerose é a principal causa de doenças cardiovasculares, que são consideradas a doença fatal em todo o mundo. A hipercolesterolemia desempenha um papel crítico no desenvolvimento de aterosclerose e doenças cardiovasculares. Muitos estudos afirmaram que os probióticos podem afetar a hipercolesterolemia via metabolismo do colesterol. Vários mecanismos explicam como os probióticos podem exercer seu efeito redutor de colesterol, estes incluem a desconjugação do sal biliar pela enzima hidrolase BSH, síntese de ácidos graxos de cadeia curta e integração de colesterol na membrana celular bacteriana. (HASSAN et al. 2019).

Os probióticos também têm efeito na doença hepática gordurosa não alcoólica (DHGNA), caracterizada por um aumento no conteúdo de gordura das células hepáticas. Os probióticos presentes na microbiota intestinal podem neutralizar a patogênese da DHGNA através do deslocamento de patógenos promotores desta doença, redução da produção geral de etanol microbiano, promoção da função da barreira intestinal e supressão de cascatas inflamatórias (ELSHAGHABEE et al., 2019).

No estudo realizado por Gomes et al. 2017, foi descrito pela primeira vez, que um mix de probiótico *(Lactobacillus acidophilus* e *casei*; *Lactococcus lactis*; *Bifidobacterium bifidum* e *lactis*; 2×10^{10} UFC/dia) reduziu a adiposidade abdominal e aumentou a atividade enzimática antioxidante de uma maneira mais eficaz do que uma intervenção dietética isolada, sem o uso de probióticos, em pacientes com excesso de peso ou obesidade.

No recente estudo de Gomes, Hoffmann, Mota, 2019, foi demonstrado que a ingestão de *Lactobacillus acidophilus* LA-14, *Lactobacillus casei* LC-11, *Lactococcus lactis* LL-23, *Bifidobacterium bifidum* BB-06 e *Bifidobacterium lactis* BL-4 não induziu uma variação global na composição filogenética da microbiota intestinal. Contudo, foi observado mudanças direcionadas em alguns grupos específicos de microrganismos com associação positiva com adiposidade, com uma redução concomitante desses microrganismos com o tratamento com probiótico.

Evidências sugerem um efeito protetor dos probióticos na prevenção de diarréia associada a antibióticos (DAA). Dentre alguns estudos em relação a probióticos, *Lactobacillus rhamnosus* ou *Saccharomyces boulardii* na concentração de 5 a 40 bilhões de UFC/dia parecem mais apropriados para prevenção de DAA em crianças recebendo antibióticos (GUO, et al., 2019). Existem evidências suficientes para concluir que a administração adjunta de probióticos está associada a um risco reduzido de DAA. (HEMPEL, et al. 2012).

Os probióticos revelam o potencial uso no controle da periodontite, mas pouco se sabe sobre suas interações com as células epiteliais gengivais (GECs). No trabalho de Albuquerque-Souza et al. 2019, é sustentada a hipótese de que os probióticos podem modular a resposta inflamatória mediada por *P. gingivalis* nos GECs. Os probióticos podem prevenir a morte celular e reduzir a adesão e invasão bacteriana, e sua capacidade de interferir na síntese de quimio / citocinas, bem como de ditar a expressão de alguns genes.

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SEGUNDA PARTE- ARTIGOS

ARTIGO 1 - Microbiological and physicochemical characteristics of naturally fermented brazilian table olives

Abstract

This work evaluated the microbial diversity and physicochemical characteristics of fresh fruits and during the fermentation of Brazilian table olives of cultivars Ascolano and Grappolo. The isolates were identified by polyphasic techniques: Matrix-assisted laser desorption / ionization time-of-flight mass spectrometry analysis (Maldi-Tof MS) and DNA sequencing. Chemical compositions were determined by high-performance liquid chromatography (HPLC). Twenty species of mesophilic bacteria, seven species of lactic acid bacteria (BAL) and fourteen species of yeast were identified. Some species prevailed over others, such as *Lactobacillus brevis, Lactobacillus paracasei, Pantoea Agglomerans, Staphylococcus warneri, Candida parapsilosis, Candida orthopsilosis, Cryptococcus flavescen, Bacillus simplex* and *Bacillus thuringiensis*. Glucose and mannitol were the main sugars present in the table olive. Acetic, citric and lactic acid were the acids detected at higher concentrations. In conclusion, the polyphasic methodology was efficiently performed to identify microorganisms; chemical analysis helped to understand the fermentation process of olives. These findings are relevant, characterizing previously unexplored Brazilian olives. Knowledge of the native microbiota present in the fruits of these olives and the species involved in fermentation and its evolution along the process may be useful in improving the quality of sensory properties and preservation. In addition, microbiota characterization may result in the isolation of possible biotechnologically important microorganisms.

Keywords: Olive growing; olive fruit, fermentation, microorganism, isolation.

Introduction

Table olives are one of the most important and well-known fermented fruits in the food industry, with a worldwide production currently exceeding 2.5 million tons per year. The elaboration of this food has a great impact on the world economy, especially in the Mediterranean countries, which are the main producers, with more than 80% of the total output being processed in these countries (IOC, 2019). However, this fruit has adapted to various regions of South America, where countries such as Brazil have favorable conditions to produce table olive and olive oil (Oliveira et al. 2012).

According to Portuguese Standard NP - 3034 (1987), "Table olives" means the product prepared from fruits of appropriate varieties of the species *Olea europaea* L., in a suitable state of maturity, subjected to treatments and operations to ensure its characteristics and good conservation.

Such preservation occurs by fermentative processes, in which the fermented table olive has a characteristic aroma resulting from the balance between a set of volatile compounds such as hydrocarbons, aldehydes, alcohols (ethanol), esters, ketones, and other compounds. The formation of these compounds is a dynamic process developed during the fermentation by the present microbiota (Sabatini et al., 2008).

The fermentation of olives can be performed in two ways: by the traditional process, through spontaneous fermentation, or by the addition of starter culture (Peres et al., 2012). For the preparation of naturally fermented olives, the harvested fruits are washed with water to remove surface dirt and placed in brine with a NaCl concentration ranging from 6% to 10% (Nychas et al., 2002). Then begins the "spontaneous" fermentation process, mostly driven by yeast, Gram-negative bacteria and lactic acid bacteria (Aponte et al., 2010, Nout, Rombouts, 2000; Oliveira et al., 2004; Piga et al., 2005; Panagou et al., 2011,).

This microbiota is determined by the substrate available for microorganism growth, salt concentration and also by temperature, pH, anaerobic or aerobic conditions, and presence of antimicrobial compounds, such as phenolic compounds (Nychas et al., 2002; Panagou et al., 2008; Tofalo et al., 2012).

Although olives are highly consumed in Brazil, around 7874 ton table olive were imported in July of 2019 (IOC, 2019), there are not studies on the microbiological and physicochemical characteristics of olives produced in this country. The objective of the

present study was to evaluate the microbial diversity and physicochemical characteristics of fresh fruits and during the fermentation of Brazilian table olives.

Material and methods

Plant material

In February 2017, olive from two table olive cultivars (Grappolo 541 and Ascolano) were collected at a suitable maturity stage for processing. The fruits were harvested at green stage from Experimental Farm of Maria da Fé - Minas Gerais, Brazil (22°18 'south latitude, 45°23' west longitude, average elevation of 1,276 m) protected by the Minas Gerais Agricultural Research Corporation (EPAMIG), immediately transported for analysis and selected for brine.

Olive processing

After washing with tap water, 1 kg of drupes of each cultivar were intended for microbiological, chromatographic and physicochemical analysis, the same amount was put into 1,5-litre glass containers filled with freshly prepared 10% (w/v) NaCl brine. The fermentation of each cultivar was performed in triplicate: three lots of olives coming from different trees in the same olive grove. Fermentations took place on the same days in the same conditions (± 25 ° C initial pH 8.3). Samples of fresh and fermenting olives at 30, 60 and 120 days of fermentation were obtained for microbiological and chromatographic analysis.

pH determination

The pH values of olive brine were measured by inserting a combined electrode (Ag / AgCl reference system) coupled to a potentiometer (DM20-Digimed, São Paulo, SP, Brazil) previously calibrated at 20 ° C, with pH 4 and pH 7 buffer solutions. Determinations were all times of fermentation and performed in triplicate.

Nutritional composition of fruits

The approximate composition of the fresh fruits of the two cultivars were studied. Using AOAC 1995 methods, moisture was determined according to method 925.40 with modifications; the ash content by weighing samples before and after heat treatment at 550 ° C in muffle for 6 hours (AOAC method 923.03); the fat content by soxhlet extraction method according to AOAC method 960.39; and protein according to AOAC method 976.05, evaluated by micro-Kjeldahl method, with nitrogen-to-protein conversion factor of 6.25. Crude fiber content was determined using AOAC official method No. 923.03 (1997).

Quantification, isolation, and phenotypic characterization of microorganisms

Aliquots (10 g) of the samples of fresh fruit and fermented table olive were added to flasks for a pre-enrichment, for isolation of yeast and mesophilic bacteria was used Y*east extract peptone glucose* (YEPG) broth (2% glucose, 1% peptone, 1% yeast extract, w / v), and incubation at 30 ° C on an orbital shaker (160 rpm) for 48 hours. For isolation of lactic acid bacteria (LAB), pre-enrichment was performed in Man Rogosa and Sharpe (MRS) broth with incubation at 37 ° C for 72 hours. Mesophilic bacteria were enumerated by spread plating on Nutrient Agar (Himedia, Mumbai, India) and lactic acid bacteria on MRS agar (Merck, Darmstadt, Germany) with added nystatin (Oxoid, England) to inhibit growth of yeasts and filamentous fungi. The yeasts were enumerated by spread plating on YEPG Agar (2% glucose, 1% peptone, 1% yeast extract, 1,5 % agar w / v, pH 3). The plates were incubated at 30 °C for mesophilic bacteria and yeasts, and 37 °C for LAB, during 48 h. The morphological characteristics of the colonies (cell size, cell shape, edge, color, and brightness) were recorded and the square root of the number of colonies counted for each morphotype was purified by streaking on new agar plates (Dias; Schwan, 2010). The pure cultures were stored in an ultra-freezer at −80 °C in the same broth culture media used for plating, containing 20% glycerol (w/w).

Maldi-tof sample preparation, measurement and data analysis

The obtained isolates (941 colonies) were grown using the culture medium and incubation conditions specific to each taxonomic group as described above. After incubation (18 at 24 hours), about 20 mg of each colony were aseptically transferred to microtubes. Each

isolate was analyzed in triplicate and the protein extraction methodology, equipment calibration and data analysis method were performed as described by Carvalho et al. (2016). The equipment used was a Maldi-tof microflex LT spectrometer (Bruker Daltonics, Bremen, Germany).

Molecular identification

Representative microorganisms from each group resulting from the Maldi-tof MS cluster were identified by sequence analysis of the 16S rRNA gene for bacteria or the internal transcribed spacer region (ITS) for yeast. DNA extraction was performed using Insta-Gene Matrix® Kit (Bio-Rad, Germany) following the manufacturer's instructions. For bacterial isolates, 16S-rRNA amplifications were performed using primers 27-F (5'-AGAGTT TGATCCTGGCTCAG-3 ') and 1512-R (5'-GGCTACCTTGTTACGACT-3') (Devereux and Willis 1995). For yeast isolates, ITS-PCR was performed with primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3 ') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Nielsen et al., 2007). The amplified PCR products were sent for sequencing to the Macrogen USA – Humanizing Genomics (MD, USA) and the sequences were compared to the GenBank database using the BLAST algorithm (National Center for Biotechnology Information, Maryland, USA).

Determination of Organic Acids and Sugars (HPLC)

Organic acids (lactic, acetic, citric, malic and succinic acids) sugars (fructose, glucose, mannitol and sucrose) were analyzed. Fruit samples were evaluated at 0, 30, 60, and 120 days of fermentation. An aliquot of each sample (3 g: only pitted pulp) was homogenized in Falcon tubes with 20 mL of Milli-Q water by vortexing at room temperature for10 min. Extracts were filtered by vacuum pump. Brine sample were evaluated at 30,60 and 120 days of fermentation. 2 mL of each sample was homogenized with 2 mL of Hexane solvent (Sigma Aldrich, Germany) by vortexing for 2 min. The solvent was discarded, and extracts were filtered by through a 0.22μm cellulose acetate membrane. For the analyses of organic acids, the supernatant pH of both samples (fruit and brine) was adjusted to 2.0 using a 200 mM perchloric acid solution and centrifuged (8000 rpm for 5 min). After acidification, the

supernatant was filtered through a 0.22μm cellulose acetate membrane, all sample were stored at−18 °C until analysis.

The extracts were analyzed using a HPLC system (Shimadzu, Japan). For the acids analysis a Shimpack SCR-101H (7.9 mm ×30 cm) column was used with a 100 mM solution of perchloric acid, with a flow rate of 0.6 mL per min as the mobile phase. The oven temperature was kept at 50 °C, detected with a 210-nm UV detector, and at 30 °C. For the analysis of sugars, a Shimpack SCR-101C (7.9 mm x 30 cm) column was used with a 100 mM solution of milliq water, with a flow rate of 0.5 mL per min as the mobile phase. The oven temperature was kept at 80 °C, detected with a refractive index detector. The quantification of compounds was performed using calibration curves constructed with different concentrations of standard compounds malic and citric acids (Merck, Germany); lactic acid (Sigma Chemical, USA); acetic and succinic acids (Sigma Aldrich, Germany); sucrose (Sigma Chemical, USA); fructose (Merck, Germany), mannitol, glucose (Vetec, Brazil); and analyzed using the same conditions as for the samples. Analyses were performed in triplicate.

Statistical analysis

Analyses of the variance and the Scott-Knott test were performed with SISVAR 5.1 software (Ferreira, 2008). A value of $P< 0.05$ was considered significant.

Results and discussion

pH and Nutritional composition

The initial pH of brine added in the olives of cultivars Ascolano and Grappolo was approximately 8.38, after 30 days of fermentation there was a significant reduction ($P <0.05$) for both cultivars, the value reached about 4.91 ± 0.5 for Ascolano cultivar and Grappolo cultivar the pH was 4.65 ± 0.23. At the end of the fermentation, after 120 days, the pH reached about 4.51±0.21 for cultivar Ascolano and 4.57±0.47 for cultivar Grappolo, with no significant difference ($P> 0.05$) between the two cultivars (Figure 1).

Similar results were reported by Pino et al. 2018, analyzing olives of Nocellara Etnea cultivar, the pH values dropped faster in the early days of fermentation and at the end of the process (after 120 days of fermentation) the pH values ranged from 4.6 to 4.2. In the work of

Tofalo et al. (2012) the pH of Italian green table olives brine ranged 4.01 and 4.62. It is well established that pH below 4.5 could preserve table olives from spoilage and pathogen growth in fermented products, such as *C. botulinum* (Perricone et al. 2010). Therefore, the pH found at the end of the fermentation process of Brazilian table olives contributes to the preservation and safety of this product.

The nutritional composition of fresh fruits olives cultivars Ascolano and Grappolo was evaluated (Table 1). There was a significant difference ($p < 0.05$) for moisture, fat and carbohydrates content among olive cultivars. The cultivar Ascolano had a higher moisture and carbohydrates content (65.73 and 88%, respectively). The other compounds (protein, ashes, fibers) analyzed were very similar between the two cultivars analyzed.

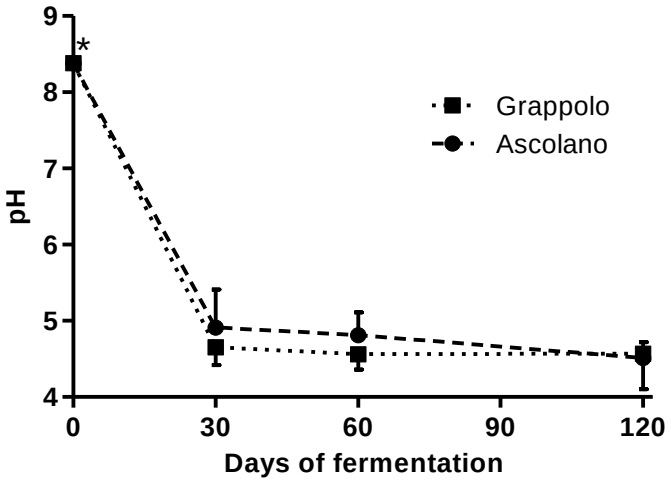


Figure 1 Changes in a pH during 120 days of brine fermentation. Error bars indicate standard errors of the means. * Indicate significant differences ($P<0.05$) at all time of fermentation by the Scott-Knott test.

Table 1 Approximate composition of fresh olives fruits of Ascolano and Grappolo cultivars

	Cultivar	
Composition (%)	Ascolano	Grappolo
Moisture	65.73 [a] ± 0.27	64.55 [b] ±0.05
Fat	15.38 [b] ± 1.19	20.15 [a] ± 0.02
Protein	3.06 [a] ± 0.24	3.61 [a] ± 0.27
Ashes	5.36 [a] ± 0.49	5.04 [a] ± 0.50
Fibers	4.37 [a] ± 0.08	4.16 [a] ± 0.26
Carbohydrates	5.88 [a] ±1.21	2.43 [b] ± 0.52

Results (% in whole matter) are expressed as mean ± SD, determined in duplicate. Different letter in the same column indicate significant differences ($P<0.05$) by Scott-Knott test

Quantification of microorganisms

The bacterial and yeast population present in the olives before and during the fermentation process were quantified by plating (Figure 2). The natural fermentation process of cultivars table olives Ascolano and Grappolo were characterized by two distinct phases. In the first phase (first 30 days) an increase in the yeast population was observed, and in general the lactic acid bacteria and mesophilic bacteria population decreased during this period. In the second phase, the mesophilic bacteria population decreased, and the lactic acid and yeast bacteria concentration increased, the latter being predominant during the whole fermentation process.

The mesophilic bacteria count started at 8.11±0.54 and 8.95±0.5 log CFU per g for cultivars Ascolano and Grappolo but decreased throughout the process until reaching values of 4.17±0.38 and 4.59±0.53 CFU per g, respectively. In contrast, total yeasts started at 5.72±0.43 and 7.18±0.21 log CFU per g for the fresh fruits of cultivars Ascolano and Grappolo, and increased throughout the fermentation process, reaching values of 9.17±0.54 and 8.06±0.37 log CFU per g, respectively. Similar behavior in population counting was observed for lactic acid bacteria in cultivar Ascolano, in which fresh fruits presented a total of 6.58±0.31 log CFU per g after 120 days of fermentation, this value increased to 8.18±0.29 log CFU per g, however, a reduction in the count of LAB for cultivar Grappolo was observed, initially the count was of 7.49±0.17 log CFU per g and at the end of the fermentation (120 days) reached 6.18±0.62 log CFU per g.

Although the mesophilic bacteria were predominant in the fresh fruits of both cultivars, throughout this fermentation process there was a reduction of this group of microorganisms, which at the end of fermentation presented a lower count when compared to the presence of yeast and lactic acid bacteria. This event probably occurs due to the sensitivity of total bacteria to the presence of acids, since the fermentation process of olives is characterized by the progressive reduction of pH values, therefore the total bacteria present tend to disappear (Garrido-fernandez et al., 1997).

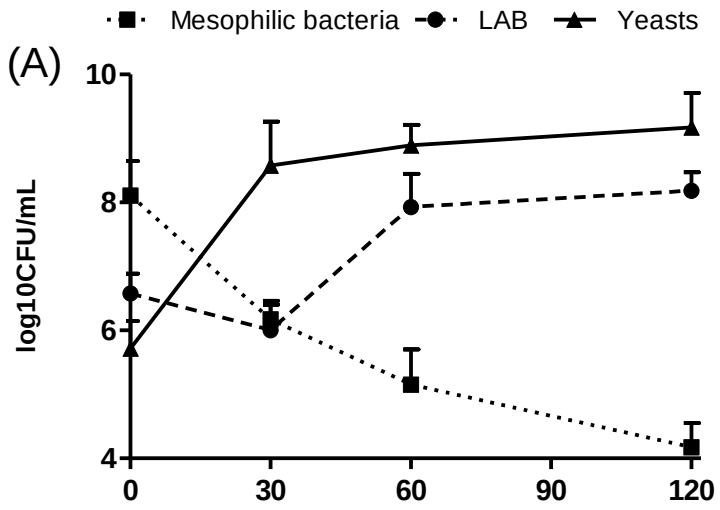
A reduction in LAB growth was observed in the first phase of cultivars Ascolano and Grappolo, this fact may be a result of the sensitivity of such microorganisms to the presence

of phenolic compounds in olive pulp and brine (Arroyo-lopéz et al., 2008), as well as the presence of high salt concentration and lower nutrient availability in the environment (Aponte et al., 2010). The LAB play an extremely relevant role in the fermentation process of olives due to their ability to produce lactic acid and antimicrobial compounds such as bacteriocins, being responsible for promoting rapid acidification of brine and consequently inhibiting the development of pathogenic microorganisms (Arroyo-lopéz, et al., 2012).

Yeasts were predominant in the fermentation process of olives cultivars Ascolano and Grappolo. Such predominance during fermentation may be related to the fact that yeasts are more tolerant to the environmental conditions often found in table olives processing, such as NaCl concentrations (8- 10% w/v) (Fernández et al., 1997; Panagou et al., 2008; Rejano et al., 2010), phenolic compounds present and low pH (Arroyo-lopéz et al., 2009).

As the results showed high yeast counts throughout the fermentation stage, they may play a significant role in table olives as they produce volatile compounds and metabolites with important organoleptic attributes that determine the quality and flavor of the final product Some species are associated with oleuropein hydrolysis, catalyzed by the enzyme glucosidase, contributing to the removal of bitterness that is naturally present in olives. (Hernández et. al., 2007; Arroyo-lopéz et al., 2008).

For Ascolano olives, a higher concentration of microorganisms was observed at all times of the fermentation process. This fact can probably be explained by the higher carbohydrate concentration (Table 1), so this fruit has the highest amount of nutrients available for the development of microorganisms.



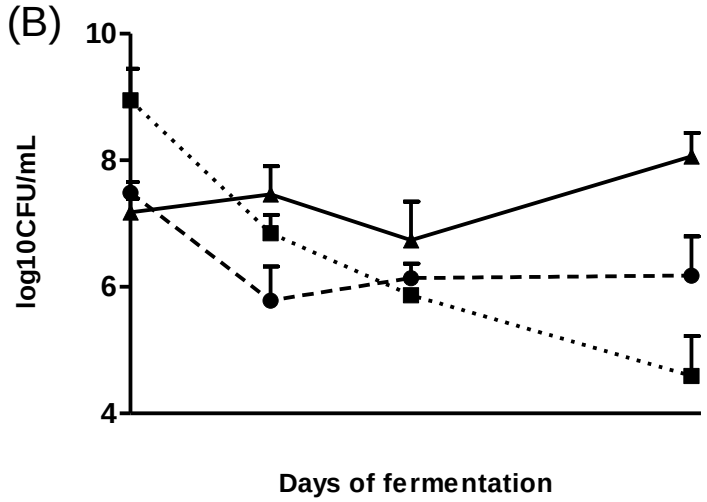


Figure 2 Changes in LAB, yeast and total bacterial populations over 120 days of fermentation. A: cultivar Ascolano, B: cultivar Grappolo. Error bars indicate standard errors of the means.

Identification of microorganisms

Twenty species of mesophilic bacteria belonging to 8 genera were identified, composed of 12 species identified in the Ascolano variety and 15 species in cv. Grappolo. The lactic acid bacterial population was less diverse in terms of species. Seven LAB species were identified, belonging to two genera. All species were identified in cultivar Ascolano, and three species identified for cultivar Grappolo. The yeast population was more diverse in terms of genera, comprising 11 genera and fourteen identified species, all identified in cultivar Ascolano and seven species (6 genera) identified in cultivar Grappolo (Table 2).

Nine hundred and forty-one isolates were obtained, and identification was performed by a combination of MALDI-TOF and 16S rRNA sequencing, among them 544 isolates correspond to cultivar Ascolano and 397 comprise cultivar Grappolo. From the analyzed sequences a similarity of 97% -100% was observed. The abundance of each microorganism was calculated in relation to the total population of the different groups (lactic acid bacteria, total bacteria and yeast) analyzed in the fruit and at each time of the fermentation process. All strains identified by sequencing were deposited with the National Center for Biotechnology Information (NCBI) and are shown in Table 2. Some of the bacteria and yeast strains

identified in the study were deposited with the Microbiology Crop Collection (http: //www.ccma.dbi.ufla.br) and were coded as CCMA (as listed below).

The genera of LAB found were *Lactobacillus* and *Enterococcus*. These genera are usually found in fermented green olives not treated with NaOH (Bravo et al., 2007; Hurtado et al., 2008; Randazzo et al. 2004). Recent works focusing on the study of bacterial biodiversity in table olives has found that *L. pentosus* and *L. plantarum* were the dominant species in LAB. (Lucena-Padrós et al., 2014a,b; Tofalo et al., 2014; Comunian et al., 2017, Benítez-Cabello et al, 2019, Lucena-Padrós, Ruiz-Barba, 2019). On the other hand, our work showed that these species did not dominate the fermentation process, being isolated only after 120 days of fermentation, the dominant LAB species in Brazilian fermented green olives not treated with NaOH were *Lactobacillus brevis* and *Lactobacillus paracasei.*

Regarding mesophilic bacteria, the identified microorganisms belong mainly to the families Enterobacteriaceae, Staphylococcaceae and Bacillaceae, previously isolated from table olives. (Campaniello et al., 2005). In agreement with this study, other authors also observed that Enterobacteriaceae members growth in olives mainly at the beginning of fermentation (De Castro et al., 2002; Tassou et al., 2010; Alves et al., 2012; Randazzo et al., 2012).

Regarding yeasts species, in the fresh olives were identified the genera *Cryptococcus* and *Rhodotorula*. According to Hernandéz et al. (2007) whatever their origin, fresh olives are usually colonized mainly by yeasts of the genera *Cryptococcus*, *Candida* and *Rhodotorula*. The presence of yeast during the processing of table olives is very common, particularly species of genera *Candida*, *Pichia*, *Rhodotorula*, *Saccharomyces* and *Debaryomyces* (Garrido-Fernández et al., 1997; Arroyo-López et al., 2012). *P. guilliermondii* is a species related to high volatile phenol production (Martorell et al., 2006; Barata et al., 2006), but has rarely been associated to olive environments (Arroyo López et al., 2007). Analyzing naturally fermented Spanish green table olives, Arroyo López et al. (2006) also identified the species *Saccharomyces cerevisiae* and *Geotrichum candidum.*

The *Candida parapsilosis* yeast was found throughout the olive fermentation process of this study, which agrees with the study performed by Aponte et al. (2010), that revealed the presence of this specie, during the entire fermentation period of Sicilian green table olives. In a recent study (Sidari, Martorana, De Bruno, 2019) the yeast biota associated with table olives of the cultivar *Nocellara messinese* naturally fermented was studied, some species in common

with this study were identified: *Saccharomyces cerevisiae, Wickeramomyces anomalus* and *Candida tropicalis*.

Olive microbiota studies mainly refer to fruits from the Mediterranean region, some isolated species in these olives are also present in Brazilian olives, but most microorganisms and their concentration along the fermentable process of Brazilian olives are quite variable. Therefore, in addition to the microbiota variation that occurs according to table olive cultivars, the amount and species of microorganisms may also vary according to the regions and climates characteristic of olive cultivation.

The species found in the two cultivars were *Lactobacillus brevis* CCMA1766 , *Lactobacillus paracasei* CCMA1763, *Lactobacillus pentosus* CCMA1768, *Bacillus altitudinis, Bacillus megaterium, Bacillus subtilis* CCMA 1781, *Pantoea Agglomerans, Staphylococcus epidermidis, Staphylococcus hominis, Staphylococcus warneri* CCMA 1778, *Candida parapsilosis* CCMA1752, *Candida orthopsilosis* CCMA1757, *Cryptococcus flavescens, Dipodascus australiensis* CCMA 1755, *Galactomyces candidum* CCMA 1744, *Meyerozyma caribbica* CCMA1758 and *Rhodotorula mucilaginosa.* Some species were found only for cultivar Ascolano, *Enterococcus casseliflavus, Enterococcus faecium, Lactobacillus plantarum, Lactobacillus vaccinostercus, Acinetobacter ursingii, Bacillus cereus* CCMA 1779, *Bacillus mojavensis, Bacillus pumilus, Curtobacterium sp, Candida tropicalis* CCMA1751, *Debaryomyces hansenii* CCMA1761, *Galactomyces geotrichum* CCMA 1759, *Geotrichum silvícola, Pichia guilliermondii* CCMA1753, *Saccharomyces cerevisiae* CCMA1746 and *Wickerhamomyces anomalus.* In cultivar Grappolo species exclusively identified were *Bacillus methylotrophicus* CCMA 1780 *Bacillus simplex, Bacillus thuringiensis, Dermacoccus nishinomiyaensis, Lysinibacillus boronitolerans, Micrococcus luteus, Staphylococcus capitis, Staphylococcus pasteuri* (Table 2).

In general, the difference in the microbiota composition present in olives can be attributed to the olive variety, the means used for harvesting and the storage conditions (Fakas et al., 2010). The olive trees analyzed in this study coexist in the same habitat, but their olives harbor different microbial species. The relationship between plant characteristics at cultivar level (and their genotypes) and microbial populations in the developing philosopher has already been observed (Balint-Kurti et al. 2010; Hunter et al., 2010).

It should be noted that the method of identification by a combination of MALDI-TOF and 16S rRNA sequencing used in this study is most likely accurate in identifying microorganisms first isolated from cv olives. Ascolano and Grappolo. A greater variety of

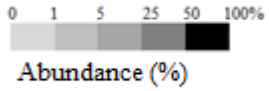
species was observed for the cultivar Ascolano, together with a higher microorganism count, so this Brazilian table olive cultivar can be further explored to obtain strains with functional potential.

Table 2 Population of bacteria and yeast present in olive fruit and during fermentation

Species identified	Log CFU/g	Accession number	Ascolano				Grappolo			
Acid lactic bactéria			**F**	**30**	**60**	**120**	**F**	**30**	**60**	**120**
Enterococcus casseliflavus	3.90	n.s.								
Enterococcus faecium	4.97	n.s.								
Lactobacillus brevis	8.21	EF412982.								
Lactobacillus paracasei	8.01	CP025582.								
Lactobacillus pentosus	6.38	KX057553.								
Lactobacillus plantarum	5.10	KJ994464.1								
Lactobacillus vaccinostercus	6.30	n.s.								
Species identified	**Log CFU/g**	**Accession number**	**Ascolano**				**Grappolo**			
Total bactéria			**F**	**30**	**60**	**120**	**F**	**30**	**60**	**120**
Acinetobacter ursingii	3.04	n.s.								
Bacillus altitudinis	6.18	n.s.								
Bacillus cereus	3.30	KJ626301.1								
Bacillus megaterium	7.56	n.s.								
Bacillus methylotrophicus	4.70	KR818074.								
Bacillus mojavensis	5.16	n.s.								
Bacillus pumilus	4.83	n.s.								
Bacillus simplex	6.85	n.s.								
Bacillus subtilis	4.82	JN984933.1								
Bacillus thuringiensis	8.72	KT340483.								
Curtobacterium sp.	5.44	n.s.								
Dermacoccusnishinomiyaensi	4.76	n.s.								
Lysinibacillus boronitolerans	4.93	n.s.								
Micrococcus luteus	4.91	n.s.								
Pantoea Agglomerans	8.14	FR832419.								
Staphylococcus capitis	4.40	n.s.								
Staphylococcus epidermidis	5.44	n.s.								
Staphylococcus hominis	5.94	n.s.								
Staphylococcus pasteuri	3.85	LN623633.								
Staphylococcus warneri	8.60	MH368264.								
Species identified	**Log CFU/ g**	**Accession number**	**Ascolano**				**Grappolo**			
Yeast Total			**F**	**30**	**60**	**120**	**F**	**30**	**60**	**120**
Candida parapsilosis	8.91	KY102320.								
Candida orthopsilosis	8.94	FM178400.								
Candida tropicalis	4.18	KP675687.								
Cryptococcus flavescens	6.13	n.s.								
Debaryomyces hansenii	4.74	KX981201.								
Dipodascus australiensis	6.00	KU132331.								
Galactomyces candidum	4.30	KM115127.								
Galactomyces geotrichum	5.00	JQ668739.1								
Geotrichum silvícola	6.71	n.s.								
Meyerozyma caribbica	7.57	KP675261.								

Pichia quilliermondii	3.40	EF190227.
Rhodotorula mucilaginosa	7.17	n.s.
Saccharomyces cerevisiae	5.36	CP029160.
Wickerhamomyces anomalus	4.15	LC120363.

Database accession number of NCBI Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi) n.s.: not sequenced, identified by MALDI-TOF with a score above 2.000; F: fresh olive fruit 30-120: Days of fermentation;



Determination of Organic Acids and sugars (HPLC)

In fresh olives, sucrose, glucose, mannitol and fructose were identified and quantified (Figure 3). These sugars are naturally present in the olive pulp due to transport by phloem from mature leaves and by photosynthesis formation, and they are very significant for the lipid biosynthesis and fruit growth. Sucrose, glucose and fructose were also identified in other studies, and were the main sugars in fresh fruit olives (Tekaya et al. 2018, Sánchez-Rodríguez et al. 2019).

The amount of total sugar at the fresh fruit was not abundant (0-2.7 g/L), and glucose was the main component in both the cultivars olive, presenting high concentration ($P<0.05$) in both fruit and brine (Figure 3). For the cultivar Ascolano, apparently the glucose and mannitol were consumed during the fermentation, presenting reduction ($P<0.05$) in concentration over the fermentation time. However, in the cultivar Grappolo, only glucose concentration decrease ($P<0.05$) over time of fermentation.

In olives and brine of our study, total sugars were not utterly exhausted at the end of fermentation. On the other hand, in a recent study (Romero-Gil et al. 2019), commercial seasoned packaged cracked olives of the *Aloreña de Málaga* cultivar were analyzed and the brines total sugars were completely depleted at the end of the shelf-life of olives. Fact also observed in the work of Tufariello et al. 2019, where in the black olive fermentations of the cultivars Kalamàta, Picual and Manzanilla, glucose was almost completely consumed in all spontaneous fermentation.

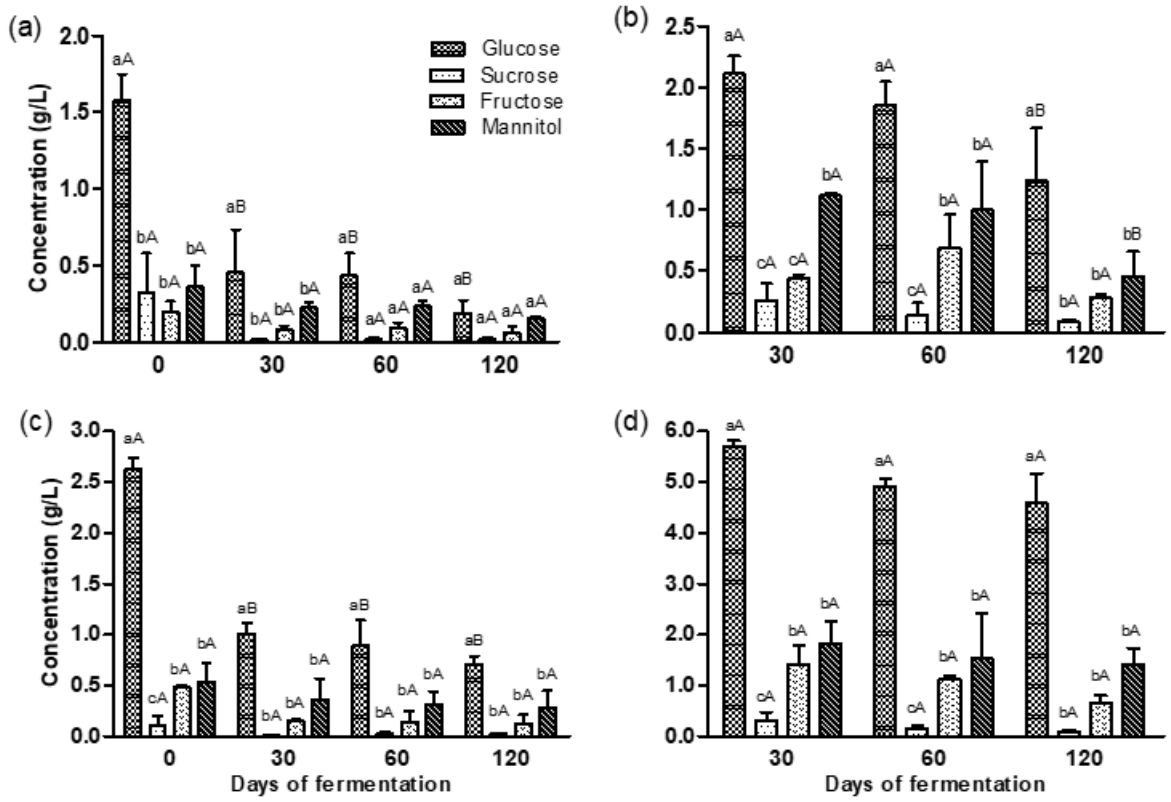


Figure 3 Changes in the sucrose, glucose, fructose, and mannitol over time during the fermentation. A: olive fruits cultivar Ascolano; B: brine cultivar Ascolano; C: olive fruits cultivar Grappolo; D: brines cultivar Grappolo. Error bars indicate standard errors of the means. Different lower-case letters in the same column indicate significant differences ($P<0.05$) in each time and different capital letters in the same column indicate significant differences ($P<0.05$) at all time of fermentation by Scott-Knott test

The acetic acid, citric acid, lactic acid, malic acid, succinic acid were quantified and identified in our study (Figure 4). These results are in line with a study of Tufariello et al, 2015, studying black olives naturally fermented, all acids were also quantified, and lactic and acetic acid increased during fermentation.

The individual and total levels of organic acids in olive fruits may change in relation to the maturation and variety (Ergonul, Nergiz, 2010). In our study, the ripening effect can be ignored because both cultivars were harvested at the same ripening point.

The organic acids (lactic, citric, tartaric and acetic acid) were the more representive metabolites in the brines, according to data produced by spontaneous fermentations performed on green and black olive fermentations (Nychas et al., 2002; Chorianopoulos et al., 2005; Panagou et al., 2008; Bleve et al., 2014, 2015a). The compounds acetic acid, succinic acid,

formic acid and ethanol are usually produced by yeasts (Querol and Fleet, 2006). In a study by Aponte et al. (2010) Sicilian table olive fermentation dominated mainly by yeasts presented a considerable number of volatile compounds during the fermentation, and the most of these compounds derived from the degradation of polyunsaturated fatty acids through lipoxygenase pathways.

The concentration of acetic acid found in Ascolano cultivar (fruit and brine) and Grappolo (fruit) olives increased ($P<0.05$) over the fermentation time, this fact was likely due to the presence of heterofermentative LAB strains (Romero-Gil, et al. 2019).

The concentration of sugars and organics acids was higher in olive brines than in fresh fruits, a fact already expected since in spontaneous fermentation occurs the diffusion of sugars and other soluble compounds through the olive peel to the brine (Hernández et. al., 2007; Arroyo-López et al., 2008).

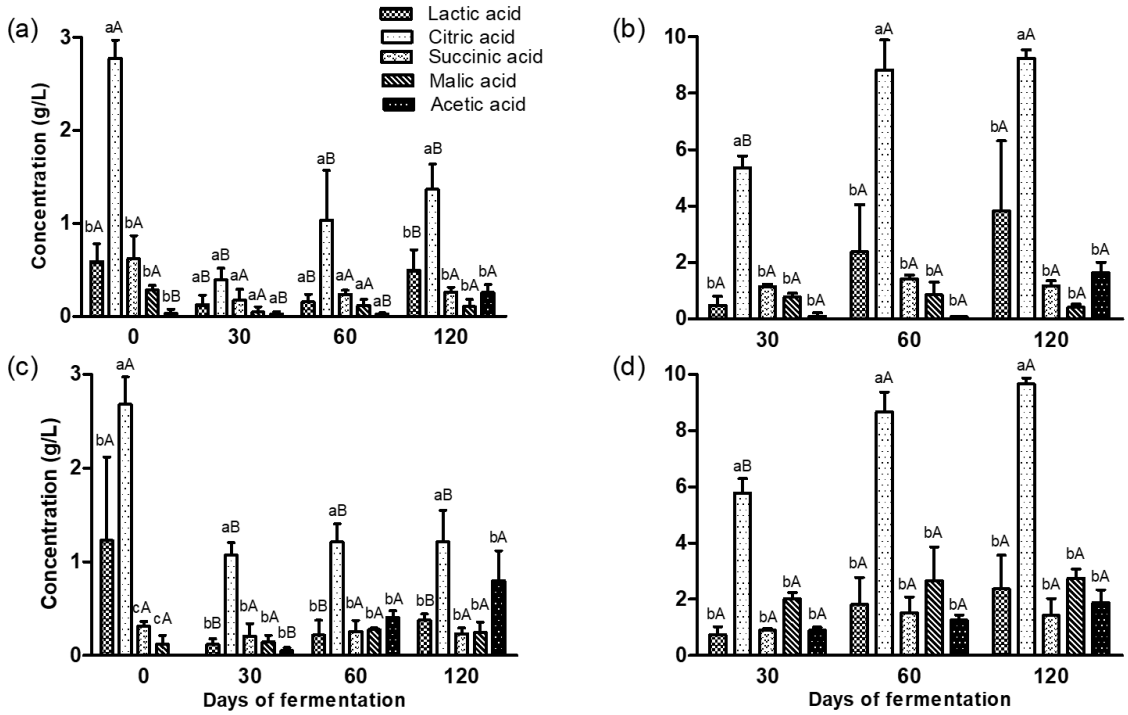


Figure 4 Changes in the acetic acid, citric acid, lactic acid, malic acid, succinic acid over time during the fermentation. A: olive fruits cultivar Ascolano; B: brine cultivar Ascolano; C: olive fruits cultivar Grappolo; D: brines cultivar Grappolo. Bar indicate SD. Error bars indicate standard errors of the means. Different lower-case letters in the same column indicate significant differences ($P<0.05$) in each time and different capital letters in the same column indicate significant differences ($P<0.05$) at all time of fermentation by Scott-Knott test.

Conclusions

Nine hundred and forty-one isolates were obtained, 20 species of total bacteria, 7 species of lactic acid bacteria and 14 species of yeast were identified. The species *Lactobacillus brevis, Lactobacillus paracasei, Pantoea Agglomerans, Staphylococcus warneri, Candida parapsilosis, Candida orthopsilosis, Cryptococcus flavescen, Bacillus simplex, Bacillus thuringiensis* showed high dominance over other species and probably have an important olive influence on fermentation.

To our knowledge, this is the first study in which the isolation and identification of microorganisms present in fresh fruits and during the fermentation process of Ascolano and Grappolo olives were carried out. Knowledge of the native microbiota present in the fruits of these olives, the species involved in fermentation and their evolution along the process, and the physicochemical characteristics may be useful in improving the quality of these olives, improving their sensory properties and preserving and safeguarding them. In addition, the characterization of the fermentation process of olives may result in the isolation of possible microorganisms with interesting biotechnological characteristics.

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Compliance with Ethical Standards

Conflicts of Interest: The authors declare that they have no conflict of interest.

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ARTIGO 2- Evaluation of Probiotic Potential of Lactic Acid Bacteria Isolated from naturally fermented brazilian table olives

Abstract

Consumption of probiotic products has recently increased with the predominant intention to promote human well-being. The selection process for new probiotic candidates is a considerable challenge to be faced. Lactic Acid Bacteria are highly valued as beneficial microorganisms for their probiotic properties. This study aimed to investigate the probiotic potential of Lactic Acid Bacteria (LAB) isolated from Brazilian naturally fermented table olives, in order to obtain new potentially probiotic strains. From a total of 14 LAB, six showed potential properties for use as probiotics: *Lactobacillus pentosus* CCMA 1768; *Lactobacillus paracasei* CCMA 1771; *Lactobacillus paracasei* CCMA 1774; *Lactobacillus paracasei* CCMA 1770; *Lactobacillus brevis* CCMA 1766 and *Lactobacillus brevis* CCMA 1762. The isolates showed a similar or higher percentage ($p<0.05$) of tolerance to pH 2.0 and bile salts (0.3% w/v), hydrophobicity, autoaggregation, coaggregation with *E. coli* and *S.* Enteritidis and *a*ntimicrobial activity against *S.* Enteritidis, *L. monocytogenes* and *S. aureus*, compared to the reference strain *Lactobacillus paracasei* LB-81. All the isolates tested showed a similar or higher percentage ($p <0.05$) of adhesion than the reference strain *L. paracasei* LBC-81, and the strain *L. brevis* CCMA 1762 showed the highest ($p<0,05$) adhesion percentage to Caco-2 and HT-29 cell lines both cells tested. These results indicate the six selected *Lactobacillus spp*. strains isolated from fermented table olives as potentially probiotic candidates.

Key words: LAB, Fermented olives, Adhesion, Hydrophobicity, Autoaggregation.

Introduction

Probiotics are live microorganisms that when administered in adequate amount confer health benefits to the host (Hill et al., 2014). Lactic acid bacteria (LAB), particularly certain strains of the genus *Lactobacillus*, are the most commonly used probiotics in foods, because they are desirable in the intestinal microflora and have "Generally Recognized as Safe" (GRAS) status (Shokryazdanet et al. 2014).

The consumption of probiotics to promote health and well-being has increased worldwide in recent years (Suez, et al. 2019). There are several mechanisms that probiotic microorganisms use to exert their beneficial effects on the host health: colonization and adhesion to epithelial cells, production of biosurfactants and antimicrobial compounds (organic acids, bacteriocins, hydrogen peroxide and other compounds), co-aggregation of pathogens, competition for nutrients and support the immune system (Pellegrino et al., 2018).

Most commercially available probiotic microorganisms are originally isolated from human feces samples, to maximize the likelihood of compatibility with the intestine microbiota and improve their chances of surviving in this inhospitable environment. However, microorganisms isolated from fermented foods have shown probiotic abilities in “in vitro” tests, therefore, traditional fermented foods, as a fermented vegetables, are a reservoir for research into new strains with new functional properties (Cruz; Faria; Dender, 2007; Rivera-espinoza; Gallardo- Navarro, 2010, Ayeni et al., 2011).

Microbial ecology studies of olive fermentative processes show a succession of mixed populations (Enterobacteriaceae, lactic acid bacteria and yeast), as well as a promising source of new probiotic lactic acid bacterial strains (De Bellis et al., 2010; Bautista-Gallego et al., 2013; Argyri et al., 2013; Doulgeraki et al. 2013; Bleve et al. 2015; Tufariello et al. 2015; Bonatsou, et al. 2017; Pino et al., 2018; Guantario et al., 2018; Benítez-Cabello et al. 2019).

Properly reducing the pH of table olives is the determining factor for successful fermentation and ensuring a safe and quality product. Due to the complexity and the exclusive dependence of the fruit's natural microbiota on pH reduction, the non-spontaneous process performed through starter culture inoculation is a widely used alternative (Ruiz-barba, Jiménezdíaz, 2012).

The use of starter crops with probiotic potential becomes a major attraction for table olives manufacturing, these microorganisms can prevent the development of spoilage through competitive inhibition, allowing a rapid pH decrease, avoiding any kind of microbiological

spoilage during fermentation. , besides characterizing the table olive product as potentially probiotic.

Table olives are considered a very interesting matrix to support the survival of strains with probiotic potential because of the presence of prebiotic substances, nutrient release from fruits and their microstructure. In addition, the rough surface of olives tends to protect microorganism from the acidic environment and favor the formation of mixed biofilms of microorganisms, such as LAB and yeast, capable of transporting probiotics to the human gastrointestinal tract (GI) (De Bellis et al., 2010; Ranadheera et al., 2010; Arroyo-López et al., 2012; Blana et al., 2014; Rodríguez-Gómez et al., 2014a,b, 2017; Argyri et al., 2015 Grounta et al., 2015).

Although a high number of probiotic LAB strains have been characterized, the search for new probiotic strains remains of interest because of the wide use possibilities of these microorganisms, especially their incorporation into food matrices (Ilha, et al. 2015). So, the aim of this study was to evaluate *in vitro* the probiotic potential of Lactic Acid Bacteria isolated from Brazilian naturally fermented table olives.

Chemicals and Culture conditions

A total of 14 (Suppl. Table I) Lactic acid bacteria (LAB) strains belonging to the Culture Collection of Agricultural Microbiology (CCMA) of the Federal University of Lavras and isolated from fermented naturally table olives from the Experimental Farm of EPAMIG (Minas Gerais Agricultural Research Company) in the city of Maria da Fé-MG, Brazil (22°18 ' south latitude and 45°23' west longitude). The LAB were preserved in Man Rogosa and Sharpe (MRS) (Oxoid, England) broth and were maintained as frozen stocks at − 80 °C in the presence of 20% (v/v) glycerol as a cryoprotective agent. For their use, each strain was cultivated in MRS broth and incubated at 37 °C for 48 h.

The pathogenic strain employed in the coaggregation and inhibitory action against pathogens assay were *Escherichia coli* (EPEC) INCQS 00181 CDC 055, *Salmonella* Enteritidis ATCC 564, *Listeria monocytogenes* ATCC 19117 and *Staphylococcus aureus* ATCC 8702. The pathogens are grown at 37° C for 24 h in BHI (Brain heart infusion, Himedia)

The reference strain selected for all assays was the lyophilized *Lactobacillus paracasei* LBC-81 (Danisco A / S, Copenhagen, Denmark) bacteria. This microorganism was reactivated in MRS broth at 37 °C for 48 h.

To perform standardization of LAB, pathogens and reference strain inoculum, a growth curve was elaborated, followed by absorbance (D.O. 600nm) and plaque counting using MRS agar for LAB and BHI agar for pathogens. The plates were incubated at 37 ° C for 48 hours and the inoculum standardized at 10^8 CFU / mL.

Tolerance to pH 2.0

The 14 isolates of LAB were tested for tolerance to acid pH, according to Ramos et al. (2013) with certain modifications, aiming to select the resistant strains at pH 2,0. Isolates were grown and centrifuged 5000 rpm for 5 min at 4 °C, the cell pellets were washed twice with phosphate buffered saline (PBS: 10 mM KH_2PO_4/K_2HPO_4 ,150 mM NaCl, pH 7), then the strains were resuspended in MRS broth with pH adjusted to 2.0 using an acid solution (1 N HCl) and incubated during 3 h at 37 °C. Samples (100 μL) were obtained at the beginning (time 0) and at the end of incubation (time 3) for determination of total viable count. Dilutions were made (up to 10^7) and cells were plated in duplicate on MRS agar in triplicates and incubated at 37° C for 48 h. The percentage of survival at pH 2 was calculated using the equation: Survival at pH 2 (%) = Final viable count (log10 CFU/mL) / Initial viable count (log10 CFU/mL)) x 100.

Bile salt tolerance

The bile salt tolerance assays were conducted according to the methodology used by Matijasic and Rogelj, 2000, with some modifications. The cells were cultivated and harvested by centrifuging 5000 rpm for 5 min at 4 °C, pellets were washed twice with Phosphate Buffered Saline (PBS: 10 mM KH_2PO_4/K_2HPO_4 ,150 mM NaCl, pH 7), so were resuspended in MRS broth containing 0.3 (v/v) of bile salt (Oxgall). In the times of 0 and 3 h of incubation at 37°C, viable counts on MRS agar plates were determined in triplicates. Plates were incubated at 37° C for 48 h. The percentage of survival at bile salt was calculated using the equation: Survival at bile salt (%) = Final viable count (log10 CFU/mL) / Initial viable count (log10 CFU/mL)) x 100.

Cell Surface Hydrophobicity

The cell surface hydrophobicity assays were performed according to Santos et al. (2015), with some modifications. The LAB were cultivated and the pellets cells were obtained by centrifugation (7000 rpm for 5 min at 4 °C), after the pellets were washed twice and resuspended in PBS (50 mM K_2HPO_4/KH_2PO_4, pH 6.5) solution to achieve an OD at 600 nm of 1.0, named A600 value (A0). The organic solvent n-hexadecane (Sigma-Aldrich, Saint Louis, USA) was mixed (1:5) with the cell suspension and vortexed during 2 min. 1 hour after incubation (37 °C), measured the A600 value (A) of the formed aqueous layer. The cell surface hydrophobicity was calculated using the equation: %H = [(A0–A)/A0] × 100; the values for A0: absorbance values measured before the extraction and A: absorbance values measured after the extraction with N-hexadecane.

Autoaggregation and coaggregation assays

Autoaggregation assays were done according to the methodology describe by Del Re, Sgorbati, Miglioli, Palenzona (2000) with certain modifications. The isolates were grown and centrifuged (5000 rpm for 15 min), after the pellets were washed three times and resuspended in Phosphate Buffered Saline (PBS: 10 mM KH_2PO_4/K_2HPO_4 ,150 mM NaCl, pH 7), to achieve viable counts of approximately 10^8 CFU/mL. 4 mL of the cell suspensions were mixed by vortexing (10 s) and incubated at room temperature during 24 h. After the incubation, the auto-aggregation was measured at 600 nm. The autoaggregation percentage is expressed as: 1- (*A*0/*A*t) x 100, where A0 represents the optical density at start of experiment and At the data after 24 h. All experiments were performed in triplicate

The method for preparing the cell suspensions for coaggregation with pathogens was the almost same as that for autoaggregation assay. Strains of tested LAB and pathogenic microorganisms: *Escherichia coli* (EPEC) INCQS 00181 CDC 055 or *Salmonella* Enteritidis ATCC 564 were mixed at equal parts (2 mL) in pairs (LAB + selected pathogen) by vortexing for 10 s. Control groups of individual microorganisms were tested as reference. The absorbance at 600 nm of the suspensions were measured right after mixing and passed 4 h of incubation at 37°C. Samples were retrieved following the procedure from the autoaggregation assay. The percentage of coaggregation was calculated using the equation of Handley et al. (1987):

Coaggregation (%) = $\frac{((Ax + Ay)/ 2) - A(x + y)}{} \times 100$

$$Ax + Ay/2$$

where x and y represent each of the two strains in the control tubes, and (x + y) the mixture.

Antimicrobial activity

Antimicrobial activity was evaluated following the methodology described by Prado et al. (2000). The LAB cells (10^8 CFU/mL), were centrifuged (8000 rpm for 5 min at 4 °C) and the supernatant was used for the diffusion technique in wells, 50 µl was added to the wells done on BHI medium containing 1 mL of the pathogen (10^8 CFU/mL). The plates were incubated at 37 °C for 48 h. How positive control was used wells with culture medium without microorganisms. The antimicrobial activity was confirmed by a growth free inhibition zones around the well.

Safety Assessment

Gelatinase Activity

Selected LAB isolates were tested for gelatinase production using tryptone-neopeptone-dextrose (TND) agar (g/L: tryptone 17.0, neopeptone 3.0, dextrose 2.5, NaCl 5.0, K2HPO4 2.5 and agar 20.0) containing 0.4% gelatin. The LABs were cultivated and 10 µL of the cells culture was plated in plates containing the medium TND (incubation 37ºC for 48 hrs). After the incubation was added a saturated ammonium sulfate solution enough to flood the petri plates. The positive reaction was defined by the development of clear zones around the spots (Gupta and Malik, 2007).

DNase Production

The LAB isolates were subjected to analysis DNase enzyme production. The strains were grown and 10 µL of the cells culture was plated in DNase agar medium (HiMedia). The plates were incubated at 37ºC for 48 hours. Then, was added an HCl (2mM) solution enough to flood the petri plates, the confirmation of a DNase enzyme production was determined by a clear zone around the colonies (Gupta and Malik, 2007).

Hemolytic Activity

The strains were tested for the hemolytic activity, 10 µL of the LAB cells culture were inoculated on culture medium “Triptone soy agar” 10% (TSA, bacto ™ Bd, USA),

supplemented with 5% (v / v) of defibrated ram's blood. The plates were incubated for 48 h at 37 ° C and the development of a clear zone of hydrolysis around the colonies was considered as a positive result and the isolates cannot be selected for potential probiotic use (Youssef et al. 2004).

Adhesion to Caco-2 and HT-29 cell Lines

Growth and Maintenance of Mammalian Cell Lines

The Caco-2 and HT 29 cells used in adhesion tests were provided by Cell Bank of Rio de Janeiro (BCRJ, Rio de Janeiro, Brazil) and all solutions used in assays were obtained from Invitrogen (GibcoNaerum, Denmark). The cells were grown in modified Eagle's minimal essential medium (MEM) added with 10% (v/v) heat-inactivated fetal bovine serum, 1×non-essential amino acids, and 0.1 mg/mL gentamicin, so were maintained at 37 °C in a humidified atmosphere of 5% CO2, with the culture medium being routinely exchanged for new media until the cells reached confluent monolayer (80-90%), then they were sub-passaged for the assays plates

Caco-2 and HT-29 cell line adhesion assay

The adhesion test to the human colon adenocarcinoma cell line (Caco-2 and HT 29) were conducted according to the methodology used by Ramos et al. 2013, with certain modifications. The cells were subcultivated ($2x10^5$ cell/mL) in 24-welltissue culture plates (Sarstedt, Germany) and grown for 21 days (cell medium changed on alternate days) at 37 °C in a humidified atmosphere of 5% CO2 to obtain differentiation.

The LAB isolates selected were cultured in MRS broth for 24 h at 37 °C and, after washing twice with phosphate buffered solution PBS (10 mM KH_2PO_4/K_2HPO_4 e 150 mM NaCl, pH 7,4), so were resuspended in the Eagle's minimal essential medium (MEM), at a concentration of about 10^8 CFU/mL, and 1ml of each strain suspension was added to the cell line culture in the each well and incubated (1:30 h, 37 °C , 5% CO_2 atmosphere). Then, the cells were washed three times with 1 mL of PBS to remove non adherent bacteria cells and lysed with 1 mL of Triton-X solution (0.1% v/v in PBS), with incubation for 10 min at 37 °C. After, the solution with released bacteria cells was serially diluted and enumerated on MRS agar. The plates were incubated at 37 °C for 48 h. Adhesion ability was expressed as the

percentage ratio between the bacteria counts initially seeded and the counts after the washing steps (CFU/mL). The assays were performed with triplicate and repeated twice.

Cumulative Probiotic Potential

The probiotic potential of the Lactobacillus isolates was assessed using 27 point scores, and the cumulative probiotic potential (CPP) was calculated as per the formula: CPP = (Observed score/Maximum score) × 100, depicted by Tambekar, Bhutada (2010). Each property of isolated probiotics from olive table, commercial probiotic preparations were scored as under:

Probiotic characters	Indication	Score
Survival at pH 2.0	87-92%	1
	93-96%	2
	97-100%	3
Survival at Bile	87-92%	1
	93-96%	2
	97-100%	3
Hydrophobicity	16-21%	1
	22-26%	2
	27-32%	3
Autoaggregation	74-79%	1
	80-84%	2
	85-89%	3
Coaggregation with *E. coli*	22-26%	1
	27-31%	2
	32-36%	3
Coaggregation with *S.* Enteritidis	53-57%	1
	58-61%	2
	62-65%	3
Activity Antimicrobial	2-2,1mm	1
	2,1-2,2 mm	2
	2,2-2,3 mm	3
Adhesion to Caco-2 cells	1,8-5,8%	1
	5,9-10,8%	2
	10,9-17,8%	3
Adhesion to HT-29 cells	6-20%	1
	21-40%	2
	41-55%	3

Statistical analyses

Analyses of the variance and the Scott-Knott test were performed with SISVAR 5.1 software (Ferreira, 2008). A value of $P< 0.05$ was considered significant.

Results and Discussion

Tolerance to pH 2.0 and bile salt

In order to confer beneficial effects on the host, it is crucial that probiotic bacteria are alive in the food to be consumed, and additionally can reach the large intestine in an adequate amount to colonize and proliferate in this environment (Shah, 2000).

A total of 14 Lactic acid bacteria (LAB) isolates obtained from naturally table olive fermentations were exposed to acid conditions (Suppl. Table I). 13 LAB isolates strains were able to tolerate low pH maintaining viability (>8 log CFU/ml) after inoculation at pH 2.0 for 3 h. Usually, *Lactobaccillus spp.* are the bacteria that are most resistant to low pH values and have great adaptation in different food matrices, especially because they are a type of species that occurs naturally in several fermented products (Tripathi, Girir, 2014). The strain *L. brevis* CCMA1766, showed higher ($P<0.05$) count than the reference strain. The isolate *L. paracasei* CCMA1775 presented a lower percentage ($P<0.05$) of viable count after 3 h of incubation, being eliminated from the next tests.

In relation to time, most LAB presented average counts, in the incubation time of 3 h, lower ($P<0,05$) to time 0 for the pH assay, however, these strains maintained greater viability than 8 log CFU/ml, normally, Although of the number of viable cells has decreased, the survival rate was quite high.

The isolates from table olive can survive the uncontrolled fermentation conditions, for natural fermentations of olive the maximum limit of pH should be 4.3 (IOC, 2004). Thus, consequently can also survive in in acidic environment.

All the tested strains exhibited bile tolerance, presenting survival rate ranged between 97,62 % (*L. paracasei* CCMA1767) and 100% (Suppl. Table II). Similar results were reported by Shokryazdanet et al. 2014, in which all *Lactobacillus* strains tested exhibited tolerance at 0.3% bile. Resistance to bile salts is considered an important criterion for selecting probiotic strains. For the selection of probiotic bacteria for human use, a concentration of 0.15-0.3% bile salt is recommended (Fuller, 2012). The outcome of bile exposure depends on the conditions a bacterial cell faces before entry into the small intestine (Begley et al., 2005).

Comparing the results of the analyzed times, all LAB presented average counts, in the incubation time of 3 h, higher or similar ($P<0.05$) to time 0 in the bile salt assay, with a high survival rate the biliary conditions.

A total of 13 LAB isolates were selected for their resistance and survival in an acidic environment, as well as for their growth in the presence of 0.3% bile salts, a similar concentration to that present in the small intestine. In a recent study (Ahmed et al. 2019) the acid tolerance at pH 2 of various *Lactobacillus* strains was between 81.68% to 85.01%, and the bile salt tolerance was of 81.96 to 84.65% revealed good survival, as our study.

Table I. Survival of LAB isolates under acid conditions

	Viable count (log10 CFU/ml) Time of exposure (h)		
Strains	0	3	Survival (%)
L. brevis CCMA1766	9.92^{aB}±0.01	11.15^{aA}±0.34	100
L. paracasei CCMA1763	9.52^{aA} ±0.02	9.97^{bA}±0.04	100
L. paracasei CCMA1764	9.86^{aA} ± 0.02	9.75^{bA}±0.49	98.85
L. pentosus CCMA1768	9.65^{aA} ±0.04	9.88^{bA}±0.14	100
L. paracasei CCMA1769	9.63^{aA} ±0.04	9.73^{bA}±0.04	100
L. paracasei CCMA1770	9.88^{aA} ±0.04	9.41^{bA}±0.34	95.32
L. brevis CCMA1762	9.96^{aA} ±0.03	8.73^{cB} ±0.05	87.65
L. paracasei CCMA1772	9.65^{aA} ±0.06	8.72^{cB} ±0.03	90.39
L. paracasei CCMA1771	9.85^{aA} ±0.13	8.64^{cB}±0.06	87.70
L. brevis CCMA1765	9.53^{aA} ±0.08	8.73^{cB} ± 0.05	91.61
L. paracasei CCMA1767	9.89^{aA} ±0.02	8.76^{cB} ±0.08	88.54
L. paracasei CCMA1773	9.87^{aA} ±0.04	8.64^{cB} ±0.06	87.54
L. paracasei CCMA1774	9.77^{aA} ±0.05	8.80^{cB} ±0.16	90.05
L. paracasei CCMA1775	9.65^{aA} ±0.06	6.04^{dB}±0.06	62.59
L. paracasei LBC-81	9.91^{aA} ±0.02	9.79^{bA}±0.12	98.79

Results Log CFU / ml are expressed as mean ± SD, determined in duplicate. Mean values of different letters, lowercase in columns and uppercase in rows differ significantly ($P <0.05$) by the Scott-Knott test.

Table II. Survival of LAB isolates under bile salt conditions

Strains code	Viable count (log10 CFU/ml) Time of exposure (h) 0	3	Survival (%)
L. brevis CCMA1766	9.67^{aA}±0.16	10.35^{aA}±0.07	100
L. paracasei CCMA1763	9.42^{aB}±0.02	10.81^{aA}±0.16	100
L. paracasei CCMA1764	9.80^{aA}±0.06	10.59^{aA}±0.16	100
L. pentosus CCMA1768	9.42^{aA}±0.06	9.38^{bA} ±0.45	99.55
L. paracasei CCMA1769	9.73^{aA}±0.04	9.70^{aA}±0.05	99.72
L. paracasei CCMA1770	9.59^{aA}±0.08	10.23^{aA}±0.11	100
L. brevis CCMA1762	9.56^{aA}±0.09	10.36^{aA}±0.48	100
L. paracasei CCMA1772	9.75^{aA}±0.05	9.74^{aA}±0.19	99.88
L. paracasei CCMA1771	9.84^{aA}±0.01	10.44^{aA}±0.41	100
L. brevis CCMA1765	9.86^{aA}±0.02	9.85^{aA}±0.16	99.89
L. paracasei CCMA1767	9.79^{aA}±0.12	9.55^{aA}±0.27	97.62
L. paracasei CCMA1773	9.81^{aA}±0.03	9.91^{aA} ±0.10	100
L. paracasei CCMA1774	9.80^{aB}±0.06	10.64^{aA}±0.29	100
L. paracasei LBC-81	9.63^{aA}±0.06	10.60^{aA}±0.18	100

Results Log CFU / ml are expressed as mean±SD, determined in duplicate. Mean values of different letters, lowercase in columns and uppercase in rows differ significantly ($P < 0.05$) by the Scott-Knott test.

Cell Surface Hydrophobicity, Autoaggregation assays and coaggregation assays

Hydrophobicity is defined as a non-specific interaction between microbial and host cells. This interaction is moderate by cell-surface proteins and lipoteichoic acids (Todorov et al., 2008). Cellular hydrophobicity is a significant property for bacteria with probiotic potential. It is often a prerequisite for probiotics to be able to adhere to the intestinal epithelium in order to colonize the gastrointestinal tract, offering beneficial effects such as the exclusion of enteropathogenic bacteria (Sharma, Sharma, Sharma, 2016). In this study, *L. brevis* CCMA1762, *L. paracasei* CCMA1773 and *L. paracasei* CCMA1770 showed significantly ($P< 0.05$) higher hydrophobicity and therefore able to interact with more cell

bodies compared to other strains, being significantly similar (P>0.05) to the reference strain *L. paracasei* LBC-81 (Suppl. Figure I).

Microbial adhesion to non-polar solvents (n-hexadecane) reflects cell surface hydrophobicity (Kos et al. 2003), hydrophobicity values ranged from 11.54% (*L. paracasei* CCMA1764) to 31.23% (*L. brevis* CCMA1762). In the work of Hernandez-Sanchez et al, 2015, was tested commercial probiotics and potential probiotics of species *Lactobacillus*, all strains showed hydrophobicity to n-hexadecane solvent <20%.

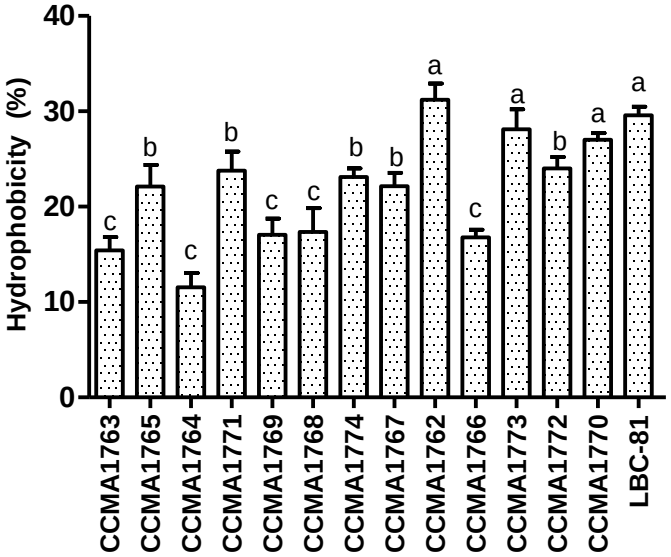

Figure I. Percentage (%) of Hydrophobicity obtained for the different LAB isolates. Bar indicate SD. Bars followed by different letters differ significantly (P < 0.05) by Scott-Knott test.

The results of the autoaggregation assay (Suppl. Figure II) indicated that all isolates presented the percentage of autoaggregation high (>60%) (Kos et al. 2003) being the highest value observed for *L. pentosus* CCMA1768 with a autoaggregation of 88.83%, similar (P>0,05) result as the one presented by the reference strain, the lowest value (P<0.05) was to the strain *L. paracasei* CCMA1764, showing only 68.19%, being this isolated excluded from the following tests.

High autoaggregation and hydrophobicity may be related to the high adhesion capacity of these microorganisms in the intestinal mucosa (Rahman et al. 2008). However, the results should be interpreted with caution, because this adherence does not necessarily mean adherence in vivo. In this work, the autoaggregation capacity had an almost normal

distribution among most strains, with values above 68%. However, hydrophobicity had a more distorted distribution, with all isolates showing values above 11%. These results are in line with a study of Bautista-Gallego et al. 2013, a study the potential probiotic properties of *Lactobacillus* spp. associated with table olives, they reported autoaggregation values higher than 50%. However, some isolates showing values above 25% of hydrophobicity.

In a previous work (Grujović, et al. 2019) the percentage of autoaggregation of species of *Lactobacillu*s ranged from 41.89 to 53.74%. The best autoaggregation ability was showed by *L. brevis* KGPMF35 isolate. On the other hand, our work showed autoaggregation of species of *Lactobacillu*s ranged from 68.19 to 88.83%, and the higher values of autoaggregation was presented by *L. pentosus and L. paracasei* isolates.

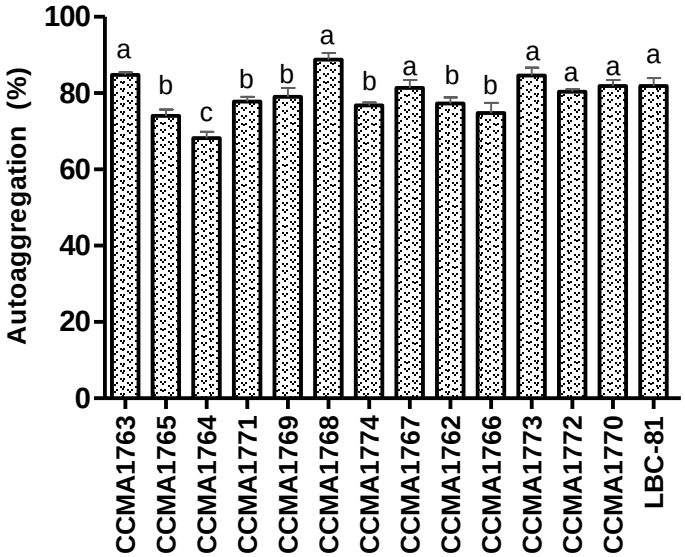

Figure II Percentage of autoaggregation of LAB isolates Bar indicate SD. Bars followed by different letters differ significantly ($P < 0.05$) by Scott-Knott test.

Probiotics can coaggregate with pathogenic microorganisms and, in turn, inhibit and kill such microorganisms by secreting antimicrobial compounds that act directly on pathogenic bacterial cells (Bao, et al. 2010). The probiotic cells bind pathogens causing a sort of clumping effect limiting the pathogen interaction with the surfaces of the host and facilitating the excretion of pathogens trough biological fluids (Janković et al., 2012). The isolate *L. paracasei* CCMA1770 showed the highest ($P<0.05$) percentage of 42.94% of coaggregation with *E. coli* (EPEC) CDC 055, and the isolates *L. paracasei* CCMA1771, *L.*

pentosus CCMA1768, *L. brevis* CCMA1762, presented the highest (P<0.05) of coaggregation with the bacteria *S.* Enteritidis *S64.*

The isolate *L. brevis* CCMA1765 showed no coaggregation with bacteria *E. coli* (EPEC) CDC 055, together with this isolate, the strains *L. paracasei* CCMA1767 and *L. paracasei* CCMA1763 presented the lower (P<0.05) percentage of coaggregation with the bacteria *S.* Enteritidis *S64.* Thus, the isolates were excluded from following tests. All the others isolate showed a similar or higher percentage (P<0.05) of coaggregation than the reference strain *L. paracasei* LBC-81 (Suppl. Figure III).

In a recently published report on the work of Gunyakti, Asan-Ozusaglam (2019) *Lactobacillus* species exhibited the highest co-aggregation with *E. coli* O157: H7 (55%), while the lowest co-aggregation was determined for *S.* Enteritidis RSKK 171 (45%), unlike this study, where the highest coagreggation was determined for *S.* Enteritidis S64 and some strains showed low or none coaggregation with *E. coli* (EPEC) CDC 055.

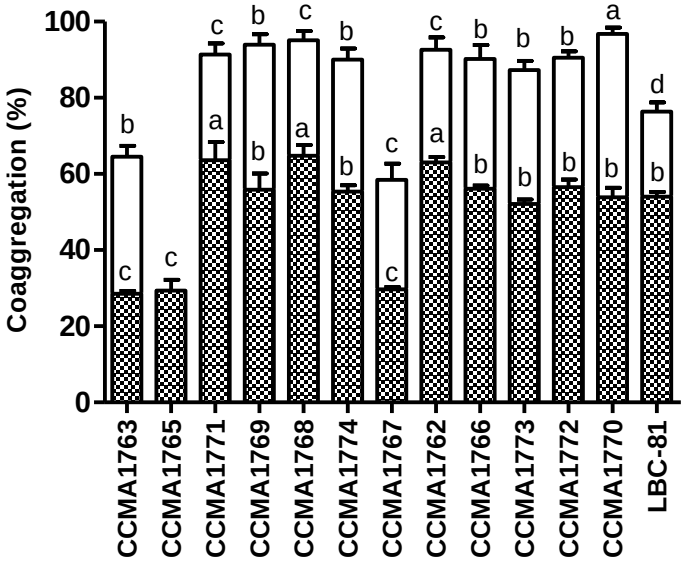

Figure III. Percentage (%) coaggregation of LAB isolates with pathogenic microorganisms. The lower histograms represent percentage coaggregation with *Salmonella* Enteritidis *S64,* and the higher histograms the value the coaggregation with *Escherichia coli* (EPEC) CDC 055. Bar indicate SD. Bars followed by different superscript letters differ significantly (p < 0.05) by Scott-Knott test.

Antimicrobial activity

The inhibitory activity of strains against the pathogenic bacteria was recorded in the range of 4,3– 9 mm (Suppl. Table III) The inhibitory activity observed against *S. aureus* ATCC 8702, all the strains showed inhibitory activity against this pathogen.

It is known that probiotics could exert competitive inhibition against pathogens, as it can confer antagonism against potentially harmful microorganism, thus contributing to prevention of their colonization on the host mucosa (Gheziel, et al. 2019). Various species of *Lactobacillus* can produce compounds with antimicrobial activities, for example organic acids (acetic and lactic acid), antifungal peptides, low-molecular-weight compounds, and antibacterial peptides (bacteriocins) (Do Carmo, et al. 2018).

The isolated *L. paracasei* CCMA1769, *L. paracasei* CCMA1772 and *L. paracasei* CCMA1773 showed no antimicrobial activity against all pathogens. Therefore, these isolates were not selected for the adhesion assays.

Gheziel, et al. 2019, studying the specie *L. plantarum*, found that the strains did not inhibit the bacteria *L. monocytogenes* CECT 4031, whereas all strains showed a strong (zone of inhibition > 6 mm) antibacterial activity against the tested *S. aureus* strains.

Table III. Antimicrobial activity of LAB isolates

	Profile pathogen inhibition		
Strains	*S. aureus* ATCC 8702	*S.* Enteritidis ATCC 564	*L. monocytogenes* ATCC 19117
L. paracasei CMA1771	++	+++	++
L. paracasei CCMA1769	+	+	-
L. pentosus CCMA1768	+	++	+++
L. paracasei CCMA1774	+++	++	++
L. brevis CCMA1762	++	++	+++
L. brevis CCMA1766	+++	++	++
L. paracasei CCMA1773	+	-	++
L. paracasei CCMA1772	+	-	+
L. paracasei CCMA1770	+++	++	++
LBC-81	++	++	++

Activity: + = presence of a clear zone of growth inhibition around well ≤ 6 mm; ++ = presence of a clear zone of growth inhibition around well ≥ 6 mm; +++ = presence of a clearly defined inhibition zone ≥ 8 mm surrounding the wells containing cell-free supernatant; – = no inhibition.

Gelatinase and Hemolytic activity, DNase Production Test

None of the strains were found to be positive for gelatinase, DNase and hemolytic activity, validating their relative safety as probiotic candidates.

Caco-2 and HT-29 cell line adhesion assay

Results observed in the Caco-2 and HT-29 cell line adhesion assay (Suppl. Figure IV) varied according to cell line, as well as between the strains employed. Isolates submitted to HT-29 cell line assay, showed the percentage of adhesion ranged from 8.02 (*L. brevis* CCMA1766) to 55% (*L. paracasei* CCMA1774), while those submitted to Caco-2 obtained inferior percentage of adhesion, between 1,84 (*L. paracasei* CCMA1771) and 17.86% (*L. brevis* CCMA1762). This result is expected, because HT-29 has a proportion of goblet cells capable of secreting mucus precursor glycoproteins. Therefore, the mucus layer is responsible for numerous ecological advantages to resident microorganisms, which includes a better fixation environment by anchor proteins. “In vitro” Caco-2 cells differentiate into monolayer homologous to intestinal epithelial enterocytes which have absorptive and non-mucosecretory characteristics (Gagnon et al. 2013).

All the isolates tested showed a similar or higher percentage ($P<0.05$) of adhesion to Caco-2 and HT-29 cells than the reference strain *L. paracasei* LBC-81. For adhesion tests with caco-2 cells, *L. brevis* CCMA 1762 strain presented the highest ($P<0,05$) adhesion rate, this same isolate also stood out in the HT-29 cell adhesion test, together with the strain *L. paracasei* CCMA 1774.

In the work of Zivkovic et. al. 2016 was observed that *L. paracasei* subsp. paracasei can biosynthesize strain-specific exopolysaccharide (EPS-SJ), and the presence of EPS on the surface of lactobacilli may improve communication between bacteria and intestinal epithelium, implying its possible role in intestinal colonization. The synthesis feature is reinforced by Bengoa et. al, 2018, studyng *L. paracasei* strains, increased capacity of adhesion to mucin and epithelial cells was observed in vitro, which is a relevant factor for the maintenance of the strain in the intestinal environment to exert its probiotic action. It has been

found that strains are polysaccharide producers, so it is possible to consider their intervention in the adhesion properties.

Regarding the strain of *Lactobacillus brevis* Hynonen et. al. (2002) states that this species are important mucosal colonizers, especially among humans, supporting their claim in the presence of the S layer, which contains a class of adhesins with affinity for various tissue targets.

In a report recently published in the work of Cao, et al. 2019, adherence to HT-29 human intestinal epithelial cells ranged from 0.72 to 27.2% for *Lactobacillus* strains. In our study, the majority of HT-29 cell line adhesion percentage remained in the same interval, two strains reached higher averages.

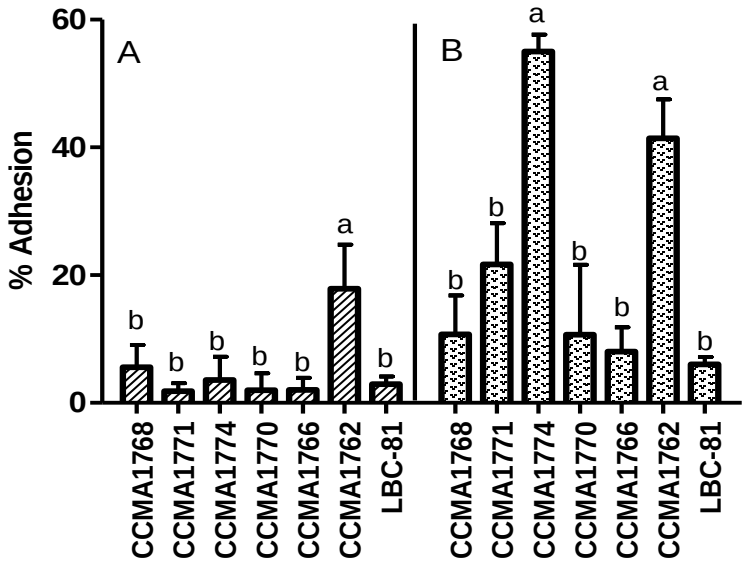

Figure IV: Adhesion capacity of LAB strains to Caco-2 (A) and HT-29 (B) cells. Bar indicate SD. Bars followed by different letters differ significantly ($p < 0.05$) by Scott-Knott test.

Calculation of probiotic potential (CPP)

The highest individual CPP for the Lactobacillus isolates were 81% for *L. brevis* CCMA1762 and 74% for the strain *L. pentosus* CCMA1768. These isolates had a higher score when compared to the other Lactobacillus strains (*L. paracasei* CMA1771 *67%*, *L. paracasei* CCMA1774 59%, *L. brevis* CCMA1766 63%, *L. paracasei* CCMA1770 70%) tested and also to the reference strain *L. paracasei* LBC-81 that showed a score of 59%. (Table IV).

The strain *L. brevis* CCMA1762 showed high score for attributes bile salt tolerance, hydrophobicity, coaggregation with *S.* Enteritidis, activity antimicrobial, adhesion to Caco-2 and HT-29 cells. The isolate *L. pentosus* CCMA1768 presented the highest score for Survival at pH 2.0, Survival at Bile, Autoaggregation and coaggregation with *S.* Enteritidis.

The demonstration of cumulative probiotic potential (CPP) of the native lactobacilli strains, as strains isolated from spontaneous fermentation of olives, has been considered as an improved criterion for the probiotic validation (Tambekar, Bhutada 2010; Gautam, Sharma, 2015; Halder et al. 2017).

With this analysis, it can be observed that all strains had a similar or higher score than the reference strain, proving the functionality of these lactic acid bacteria in relation to their probiotic potential. These microorganisms may have biotechnological application and may be used in food as starters cultures.

Table IV Cumulative probiotic potential (CPP) score card for the LAB strains

Probiotic Characters	*L. paracasei* CMA1771	*L. pentosus* CCMA1768	*L. paracasei* CCMA1774	*L. brevis* CCMA1762	*L. brevis* CCMA1766	*L. paracasei* CCMA1770	*L. paracasei* LBC-81
Survival at pH 2.0	1	3	1	1	3	2	3
Survival at Bile	3	3	3	3	3	3	3
Hydrophobicity	2	1	2	3	1	3	3
Autoaggregation	1	3	1	1	1	2	2
Coaggregation with *E. coli*	2	2	3	2	3	3	1
Coaggregation with *S.* Enteritidis	3	3	1	3	1	1	1
Activity Antimicrobial	3	2	1	3	3	3	1
Adhesion to Caco-2	1	2	1	3	1	1	1
Adhesion to HT-29	2	1	3	3	1	1	1
Total score	18	20	16	22	17	19	16
CPP for the LAB	67%	74%	59%	81%	63%	70%	59%

Conclusion

Table olives Brazilian have adequate nutritional conditions for the survival of microorganisms with probiotic potential. From a total of 14 LAB isolate from table olives, 6 showed potential properties for use as probiotics: *Lactobacillus pentosus* CCMA 1768; *Lactobacillus paracasei* CCMA 1771; *Lactobacillus paracasei* CCMA 1774; *Lactobacillus paracasei* CCMA 1770; *Lactobacillus brevis* CCMA 1766 and *Lactobacillus brevis* CCMA 1762. The results from this study revealed high cell hydrophobicity, autoaggregation, coaggregation with pathogens, tolerance at low pH, bile salts, and antimicrobial activity against pathogens. Further the showed a similar or higher percentage ($p < 0.05$) of adhesion to Caco-2 and HT-29 cells than the reference strain *L. paracasei* LBC-81.

The exploitation of national fermented products is relevant for the isolation of species with probiotic potential, the GRAS status genus *Lactobacillus* allows to consider their application for the development of new foods and probiotic products added value. In addition, "in vivo" studies should also be performed to confirm its potential benefit to human health.

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Compliance with Ethical Standards

Conflicts of Interest: The authors declare that they have no conflict of interest.

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ARTIGO 3- Probiotic properties of yeast isolated from brazilian naturally table olive fermentations

Abstract

Microbial probiotic has been used to improve the health and wellness of people. Some studies have shown that yeasts when ingested have a positive effect on the host's health and physiology and can therefore be used as probiotics. This study aimed to investigate the probiotic potential of yeasts isolated from Brazilian naturally fermented table olives, in order to obtain new potentially probiotic strains. From a total of 18 yeasts tested, 6 showed potential properties for use as probiotics: *Saccharomyces cerevisiae* CCMA1746, *Pichia guilliermondii* CCMA1753, *Candida orthopsilosis* CCMA1748, *Candida tropicalis* CCMA1751, *Meyerozyma caribbica* CCMA1758, and *Debaryomyces hansenii* CCMA1761. These yeasts demonstrated a good resistance to temperature of 37 ° C, pH 2.0, bile salts and *in vitro* digestion. Further, the yeasts also exhibited antimicrobial activity against pathogens. Regarding the surface properties, the isolates showed autoaggregation capacity, coaggregation with *E. coli* and *S.* Enteritidis pathogen, adhesion to Caco-2 and HT-29 cells, and as well as able to inhibit pathogen adhesion. Therefore, the isolated yeasts Brazilian fermented table olives present probiotic characteristics, revealing a promising potential for the development of new foods and probiotic products.

Keywords: Fermented olives. Probiotic potential. Autoaggregation. Coaggregation.

Introduction

In the naturally table olive fermentation process, lactic acid bacteria (BAL) play a essential role, especially in relation to food safety, are able to promote pH reduction and produce antimicrobial substances. (Garrido-fernandez et al., 1997; Ruiz-barba, Jimenez-diaz, 1995; Corsetti et al., 2012). In addition to BALs, yeasts are commonly found in table olive, plays a very important role because it is associated with the production of alcohols, ethyl acetate, acetaldehyde and organic acids, compounds that are relevant to the development of flavor and aroma and the preservation characteristics of table olives (Hernández et al., 2007; Arroyo-López et al., 2008).

In the last years, there has been a growing interest in the role of yeast during table olive processing due to its biotechnological and probiotic potential, existing several studies in relation an olive table from Grecee (Bleve et al., 2015; Bonatsou et al., 2018), Italy (De Angelis et al., 2015; Porru, et al 2018; Tufariello, et al. 2019; Sidari, Martorana, De Bruno, 2019), Spain (Benítez-cabello, et al, 2019) and Portugal (Pires-cabral, et al. 2018). However, data about the probiotic potential of yeasts isolated from fermented table olives from Brazil are still scarce.

Most commonly marketed probiotics are of bacterial origin, mainly the genera *Lactobacillus* and *Bifidobacterium*, when compared to the number of bacteria, a smaller amount of yeast are explored in relation to probiotic potential, the *Saccharomyces cerevisiae* var. boulardii and *Kluyveromyces fragilis* (B0399) are the only probiotic yeast species commercially available for human use (Chen, et al. 2014; Czerucka, Piche, Rampal, 2007).

The yeasts drew attention to their possible use as probiotics, due to their ability to survive during passage by human gastrointestinal tract (GIT), and also to tolerate low pH and bile salt conditions (Lourens-hattingh; Viljoen, 2001). Existing also some advantages of using yeast over bacteria like probiotics, the yeasts are normally resistant to antibiotics, staying viable in the intestinal biota during antibiotic treatments. In addition, the yeasts cannot spread the genes of antibiotic resistance and their translocation has never been reported (Czerucka; Piche; Rampal, 2007).

The growing interest in probiotics is promote both by consumer enthusiasm for marketed products with potential health benefits and by researchers inspired by the potential to prevent and treat disease (Perry, Doron, 2018). For this reason, it is necessary to go deeply in the study of yeasts as probiotics, for verifying their interesting properties. These yeast strains could be used as culture starters for the development of new functional products. Thus,

the aim of this study was to evaluate *in vitro* the probiotic potential of yeasts isolated from Brazilian naturally fermented table olives.

Materials and Methods

Yeast and bacteria Strains and Growth Conditions

A total of 18 (Table 1) yeast strains belonging to the Culture Collection of Agricultural Microbiology (CCMA) of the Federal University of Lavras and isolated from fermented naturally table olives from the Experimental Farm of EPAMIG (Minas Gerais Agricultural Research Company) in the city of Maria da Fé-MG, Brazil. The site is located at 22°18 'south latitude and 45°23' west longitude, average elevation of 1,276 m .The yeasts were preserved in YEPG (1% w/v peptone, 1% w/v yeast extract, 2% w/v glucose) broth and were maintained as frozen stocks at − 80 °C in the presence of 20% (v/v) glycerol as a cryoprotective agent. For their use in all assays, each strain was cultivated in YEPG broth and incubated at 30 °C for 48 h.

The pathogenic strain employed in the coaggregation and inhibitory action against pathogens assay: *Escherichia coli* (EPEC) INCQS 00181 (CDC 055), *Salmonella* Enteritidis ATCC 564, *Listeria monocytogenes* ATCC 19117 and *Staphylococcus aureus* ATCC 8702, All the pathogens were grown in BHI (Brain heart infusion, Himedia) broth at 37 °C for 24 h.

The reference strain selected for all assays was the lyophilized *Saccharomyces boulardii* (Floratil®, Merck, Darmstadt, Germany) yeast. This microorganism was reactivated in YEPG broth at 30 °C for 24 h.

Tolerance to pH 2.0 and Temperature of 37 °C

The 18 isolates were subjected to tolerance tests for pH 2.0 and 37 °C according to Ramos et al. (2013) with some modifications, aiming to select the resistant strains for further studies. Yeast cells were cultivated and centrifuged (5000 rpm for 5 min at 4 °C), next the strains were resuspended in YEPG broth with pH adjusted to 2.0 using an acid solution (1 N HCl) and incubated for 3 h at 37 °C. Tubes containing YEPG broth pH 6.5 were used as controls. Samples (100 µL) were obtained at intervals (0 and 3 h) for determination of total viable count. Dilutions were made (up to 10^5) and cells were plated in duplicate on YEPG agar in triplicates. Plates were incubated at 30° C for 48 h before enumeration.

Bile salt tolerance

The bile salt tolerance was investigated according to Matijasic and Rogelj, 2000, with certain modifications. Bile containing YEPG broth was prepared by the addition of 0.3 (v/v) of bile salt (Oxgall). Yeast cells were cultivated and centrifuged (5000 rpm for 5 min at 4 °C), next the strains were resuspended in YEPG broth lacking (control) or containing bile salt. After 0 and 3 h of incubation at 37°C, viable counts on YEPG agar plates were determined in triplicates. Plates were incubated at 37° C for 48 h before enumeration.

Cell Surface Hydrophobicity

Evaluations of the cell surface hydrophobicity were conducted according to the methodology used by Santos et al. (2015), with some modifications. Yeast cells cultivated were centrifuged (7000 rpm for 5 min at 4 °C), washed twice and resuspended in PBS (50 mM K_2HPO_4/KH_2PO_4, pH 6.5) to achieve an OD at 600 nm of 1.0 (A0). The organic solvent N-hexadecane (Sigma-Aldrich, Saint Louis, USA) was mixed (1:5) with the cell suspension and vortexed for 2 min. After 1 hour of incubation at 37 °C, the absorbance at 600nm (A) of the formed aqueous layer was measured. The cell surface hydrophobicity was calculated using the equation:

$$\%H = [(A0–A)/A0] \times 100$$

the values for A0 and A are the absorbance values acquired before and after the extraction with N-hexadecane, respectively.

Autoaggregation and coaggregation assays

Autoaggregation assays were performed according to Kos et al. (2003) with certain modifications. Yeasts were grown and the cells were obtained by centrifugation (5000 rpm for 15 min), washed twice and resuspended in saline PBS (50 mM K_2HPO_4/KH_2PO_4, pH 6.5) to achieve viable counts of approximately 10^8 CFU/mL. 4 mL of cell suspensions were mixed by vortexing for 10 s and autoaggregation was determined during 4 h of incubation at 30°C. Every hour 0.2 mL of the upper suspension was transferred to 96-well polystyrene microplates and the absorbance (A) was measured at 600 nm. The autoaggregation percentage is expressed as:

$$1- (A0/At) \times 100,$$

Where *At* is the absorbance at time t= 1, 2, 3, or 4h and *A0* the absorbance at t=0.

The method for preparing the cell suspensions for coaggregation with pathogens was the same as that for autoaggregation assay. Strains of tested yeast and pathogenic microorganisms: *Escherichia coli* (EPEC) INCQS 00181 CDC 055 or *Salmonella* Enteritidis ATCC 564 were mixed at equal parts (2 mL) in pairs (yeasts + selected pathogen) by vortexing for 10 s. Control groups of individual microorganisms were tested as reference. The absorbance at 600 nm of the suspensions were measured right after mixing and passed 4h of incubation at 37°C. Samples were retrieved following the procedure from the autoaggregation assay. The percentage of coaggregation was calculated using the equation of Handley et al. (1987):

$$\text{Coaggregation (\%)} = \frac{((Ax + Ay)/2) - A(x + y)}{Ax + Ay/2} \times 100$$

where x and y represent each of the two strains in the control tubes, and (x + y) the mixture.

Antimicrobial activity

Antimicrobial activity was evaluated by diffusion technique in wells, following the methodology described by Prado et al. (2000). The yeasts cells cultivated (10^7 cell/mL), were centrifuged (8000 rpm for 5 min at 4 °C) and the supernatant was used for the test, 50 µl was added to the wells done on BHI medium containing 1 mL of the pathogen (10^8 CFU/mL). The plates were incubated at 37 °C for 24 h. Wells with 50 µL culture medium without microorganisms were used as positive control. Antimicrobial activity was recorded as growth free inhibition zones around the well.

Safety Assessment

Gelatinase Activity

Gelatinase production by selected yeast isolates was studied by using tryptone-neopeptone-dextrose (TND) agar (g/L: tryptone 17.0, neopeptone 3.0, dextrose 2.5, NaCl 5.0, K2HPO4 2.5 and agar 20.0) containing 0.4% gelatin. The yeasts cells were cultivated and 10 µL of the cells culture was plated in medium TND and incubated at 37ºC for 48 hrs. Added a saturated ammonium sulfate solution enough to flood the petri plates. Development of clear zones around the spots indicated a positive reaction (Gupta and Malik, 2007).

DNase Production Test

The yeasts cells were grown and 10 µL of the cells culture was plated in plates containing DNase agar medium (HiMedia) to check production of DNase enzyme. The plates were incubated at 30ºC for 48 hours. After incubation, added an HCL (2mM) solution enough to flood the petri plates, a clear zone around the colonies was considered positive for DNase production (Gupta and Malik, 2007).

Hemolytic Activity

Hemolytic activity was determined by inoculating 10 µL of the yeast cells culture on culture medium "Triptone soy agar" 10% (TSA, bacto ™ Bd, USA), supplemented with 5% (v / v) of defibrated ram's blood. After incubation for 48 h at 37 ° C the development of a clear zone of hydrolysis around the colonies was considered as a positive result and cannot be selected for potential probiotic use (Youssef et al. 2004).

Survival of yeasts during in vitro digestion

The yeasts selected were exposed to the simulated gastrointestinal conditions according to de Albuquerque et al., 2017. The test was performed in an incubador at 37 °C and mechanical agitation was used to simulate the peristaltic movements, with rotation adjustment in each phase (esophagus-stomach, duodenum and ileum). For the simulation of the esophagus-stomach used 25 mg of pepsin diluted in 1 mL of 0.1 mM/L HCl, added at a rate of 0.05 mL/mL, pH with gradual decrease using 1 mM/L HCl (pH 5.5/10 min; pH 4.6/ 10 min; pH 3.8/10 min; pH 2.8/20 min; pH 2.3/20 min and pH 2.0/ 20 min) with agitation (130 rpm). For the simulation of the duodenal conditions used 2 g pancreatin/L of 0.1 mM/L $NaHCO_3$ and 12 g bovine bile salts/L of 0.1 mM/L $NaHCO_3$, pH adjusted for 5.0 with 0.1 mM/L $NaHCO_3$ and exposure time of 30 min with agitation (45 rpm); and ileal conditions with pH adjusted to 6.5 using 0.1 mM/L $NaHCO_3$, exposure time of 60 min with agitation (45 rpm). All the enzymes and bovine bile salts were provided by from Sigma Aldrich (St. Louis, USA). After each phase of the simulation, serial dilutions were made in sterile saline (0.9% NaCl) and inoculated YPD medium for plate count in order to enumerated viable cells.

Adhesion of Yeasts to Caco-2 and HT-29 cell Lines

Growth and Maintenance of Mammalian Cell Lines

The Caco-2 and HT 29 cells provided by the Cell Bank of Rio de Janeiro (BCRJ, Rio de Janeiro, Brazil) were grown in modified Eagle's minimal essential medium (MEM) added with 10% (v/v) heat-inactivated fetal bovine serum, 1×non-essential amino acids, and 0.1 mg/mL gentamicin. All solutions were obtained from Invitrogen (GibcoNaerum, Denmark). The cells were maintained at 37 °C in a humidified atmosphere of 5% CO2. The culture media were changed routinely, and once the cells reached subconfluence (80–90%), they were subpassaged.

Caco-2 and HT-29 cell line adhesion assay

For the adhesion test to the human colon adenocarcinoma cell line, Caco-2 and HT 29 were investigated for the 8 selected yeast isolates according to Ramos et al. 2013, with minor modifications. The cells were subcultivated (2×10^5 cell/mL) in 24-welltissue culture plates (Sarstedt, Germany) and grown at 37 °C in a humidified atmosphere of 5% CO2 for 14 days to obtain differentiation in cell media. The cell culture medium was changed on alternate days.

For the adhesion assay, yeasts were cultured in YEPG broth for 24 h at 30 °C and, after washing twice with phosphate buffered solution (PBS) solution, were resuspended in the cell line media (described in Growth and Maintenance of Mammalian Cell Lines) at a concentration of about 10^7 cells/mL. 1 ml of each yeast suspension was added to the cell line culture in the well and incubated for1 h at 37 °C in a 5% CO2 atmosphere. So, the cells were washed three times with 1 mL of PBS to remove non adherent yeast cells and lysed with 1 mL of Triton-X solution (0.1% v/v in PBS). After 10 min at 37 °C, the solution with released yeast cells was serially diluted and enumerated on YPG agar. The plates were incubated at 30 °C for24 h. Adhesion ability was expressed as the percentage ratio between the yeast counts initially seeded and the counts after the washing steps (cells/mL). Experiments were performed with triplicate determinations and repeated twice.

Inhibition of pathogenic bacteria adhesion to Caco-2 by yeasts

The inhibition of the pathogens *E. coli* and *S.* Enteritidis adhesion to Caco-2 cells by potential probiotic yeasts was performed according to Gueimonde et al. (2006) with some modifications.

Yeast (10^7 cells/mL) and pathogen (10^8 CFU/ml) were resuspended in 1 ml MEM medium (without fetal bovine serum and antibiotics) and were then added to the Caco-2 cells after 14 days of culture (differentiated cells) in 24 well plates according to treatments: 1) inoculation of bacteria (10^8 CFU/mL) for 90 minutes (control); 2) inoculation of yeasts (10^7 cells/mL) for 30 min and subsequent incubation of bacteria (10^8 CFU/mL) for additional 90 min (exclusion); 3) simultaneous inoculation of yeasts (10^7 cells/mL) and bacteria (10^8 CFU/mL) for 90 minutes (competition). After the incubation, the cells were washed three times with PBS to remove non-adhered cells and then recovered by treatment with 1% (v/v) Triton-X for 10 min at 37 ° C. Yeast and bacteria were enumerated by plating in their respective media. The ability of a pathogenic strain to adhere to Caco-2 cells in the absence (Np) and presence (Nmix) of a yeast strain was compared according to Son et al. 2017, as follows: inhibition ability = ((Np-Nmix) / Np) x100. The experiment was carried out in triplicate.

Statistical analyses

Analyses of the variance and the Scott-Knott test were performed with SISVAR 5.1 software (Ferreira, 2008). A value of $P< 0.05$ was considered significant.

Results and Discussion

Tolerance to pH 2.0, temperature of 37 °C and bile salt

The probiotics are commonly administrated orally, so they must have the ability to survive passage through the stomach and small intestine. Thus, resistance to the low pH (gastric juice) in the stomach and the presence of bile salt in the small intestine is one of the significant selection criteria for probiotic (Olejnik et al., 2005). Furthermore, such microorganisms with probiotic characteristics should survive a body temperature of approximately 37 ° C, the most strains in our study resisted this temperature, only the isolates *Galactomyces geotrichum* CCMA 1759 and *Dipodascus australiensis* CCMA 1755 did not survive the temperature of 37ºC, being excluded from the next tests.

A total of 18 yeasts isolates obtained from naturally table olive fermentations spontaneous were exposed to acid similar conditions (Table 1). Most strains were able to tolerate low pH maintaining viability after 3 hours. The strains *Galactomyces candidum* CCMA1760 and *Candida orthopsilosis* CCMA1757 showed higher (P<0,05) count than the reference strain. The isolates *Candida parapsilosis* CCMA1745, *Candida orthopsilosis* CCMA 1754 and *Candida orthopsilosis* CCMA 1747 presented a lower percentage (P<0,05) of acid ph survival after 3 h of incubation, being eliminated from the next tests.

Yeasts are present in the stomach and colon due to their resistance under varying pH conditions. Most of them can grow in the pH range from 3 to 8, and certain yeast can be resistant in greater acidic conditions (pH<1.5 for example). This is a good characteristic for considering yeasts as probiotics, furthermore the yeast could be also resisting the presence of gastrointestinal enzymes, organic acids, bile salts and changes in temperature of the environment (Czerucka et al., 2007). In this study all the tested strains exhibited bile tolerance, presenting survival rate ranged between 92,73 % (*Pichia guilliermondii* CCMA1753) and 100% (Table 2). For the selection of probiotic for human use, a concentration of 0.15-0.3% bile salt is recommended (Fuller, 2012). The bile salts are released into the duodenum after food ingestion and have antimicrobial activity against different microorganism (Reshetnyak, 2015). In relation to time, most yeasts presented average counts, in the incubation time of 3 h, higher or similar to time 0, for both assays. A total of 15 yeasts isolates from naturally Brazilian table olive fermentations were selected to the next tests by their resistance and survival in an acidic environment, as well as for their growth in the presence of 0.3% bile salts, a similar concentration to that present in the small intestine. The resistance in acidic environment not surprising, as table olive fermentations are characterized by low pH, for natural fermentations the maximum limit of pH should be 4.3 (IOC, 2004). The bile salt resistance plays an important role in physiological function regarding the survival of yeast in the intestine (Sakandar, et al. 2018). Therefore, the isolates from table olive can survive the inhospitable fermentation conditions, and consequently can also survive the passage through the GIT.

Table 1. Survival of yeasts isolates under acid conditions

Strains	Viable count (log10 CFU/ml) Time of exposure (h)		Survival (%)
	0	3	
S. cerevisiae CCMA1746	7.37^{aA} ±0.24	7.35^{cA} ±0.01	99.70
G. candidum CMA1745	7.00^{bB} ±0.12	7.29^{cB} ±0.21	100
C. orthopsilosis CCMA1749	7.58^{aA} ±0.04	6.88^{cB} ±0.74	82.83
C. parapsilosis CCMA1752	7.53^{aA} ±0.02	7.06^{cB} ±0.02	93.77
P. guilliermondii CCMA1753	7.83^{aA} ±0.07	7.02^{cB} ±0.03	92.23
C. orthopsilosis CCMA1757	7.04^{bC} ±0.06	8.59^{aA}±0.02	100
C. orthopsilosis CCMA 1776	7.64^{aA} ±0.04	7.91^{bA} ±0.01	100
C. orthopsilosis CCMA1748	7.39^{aB} ±0.55	7.94^{bA} ±0.08	100
C. orthopsilosis CCMA1750	7.01^{bB} ±0.02	7.86^{bA}±0.14	100
C. tropicalis CCMA1751	7.74^{aA} ±0.06	7.57^{bA} ±0.04	97.86
C. parapsilosis CCMA 1777	7.44^{aB} ±0.19	7.45^{cB} ±0.26	100
C. parapsilosis CCMA1756	7.31^{bA} ±0.02	6.70^{cA}±0.43	86.48
M. caribbica CCMA1758	7.80^{aA} ±0.07	7.65^{bA} ±0.01	98.10
G. candidum CCMA1760	7.02^{bB}±0.03	8.01^{aA}±0.03	100
D. hansenii CCMA1761	7.02^{bA} ±0.03	7.13^{cA} ±0.07	100
C. orthopsilosis CCMA 1747	7.18^{bB} ±0.25	5.20^{eC} ±0.29	72.50
C. parapsilosis CCMA1745	7.11^{bA} ±0.15	6.32^{dB}±0.34	88.87
C. orthopsilosis CCMA 1754	7.00^{bA}±0.08	6.46^{dB}±0.23	88.04
S. boulardii Floratil®	7.53^{aA}±0.14	7.99^{bA}±0.07	100

Results Log CFU / ml are expressed as mean±SD, determined in duplicate. Mean values of different letters, lower case in columns and uppercase in rows differ significantly ($P < 0.05$) by the Scott-Knott test. Survival at pH 2(%) = Final viable count (log10 CFU/ml) / Initial viable count (log10 CFU/ml)) x 100

Table 2. Survival of yeasts isolates under bile salt condition

Strains code	Viable count (log10 CFU/ml) Time of exposure (h) 0	3	Survival (%)
S. cerevisiae CCMA1746	7.73^{aB}±0.05	7.98^{aA}±0.01	100
G. candidum CCMA1745	7.09^{bD}±0.01	7.69^{aB}±0.02	100
C. orthopsilosis CCMA1749	7.14^{bA}±0.06	7.48^{bA}±0.53	100
C. parapsilosis CCMA1752	7.15^{bB}±0.05	8.16^{aA}±0.12	100
P. guilliermondii CCMA1753	7.57^{aB}±0.05	7.05^{cC}±0.01	92.73
C. orthopsilosis CCMA1757	7.57^{aB}±0.04	7.94^{aA}±0.02	100
C. orthopsilosis CCMA 1776	7.57^{aB}±0.04	7.94^{aA}±0.02	100
C. orthopsilosis CCMA1748	7.77^{aA}±0.1	7.81^{aA}±0.01	100
C. orthopsilosis CCMA1750	7.61^{aA}±0.13	7.39^{bB}±0.04	94.79
C. tropicalis CCMA1751	7.79^{aA}±0.01	7.45^{bB}±0.02	100
C. parapsilosis CCMA 1777	7.00^{bC}±0.01	7.53^{bB}±0.07	97.55
C. parapsilosis CCMA1756	7.47^{aA}±0.26	7.38^{bA}±0.39	96.43
M. caribbica CCMA1758	7.80^{aA}±0.07	7.70^{aA}±0.03	99.37
G. candidum CCMA1760	7.77^{aB}±0.1	7.74^{aB}±0.03	98.66
D. hansenii CCMA1761	7.71^{aB}±0.01	7.43^{bC}±0.15	96.25
S. boulardii Floratil®	7.52^{aB}±0.05	7.89^{aA}±0.01	100

Results Log CFU / ml are expressed as mean±SD, determined in duplicate. Mean values of different letters, lowercase in columns and uppercase in rows differ significantly ($P < 0.05$) by the Scott-Knott test. (Survival at bile salt (%) = Final viable count (log10 CFU/ml) / Initial viable count (log10 CFU/ml)) x 100

Cell Surface Hydrophobicity

Hydrophobic cell surface was demonstrated (Figure 1) by adherence to N-hexadecane, a non-polar solvent. Most isolates had a higher ($P<0.05$) hydrophobicity percentage than the reference strain, highlighting the isolate *Debaryomyces hansenii* CCMA1761 with 92,25% of hydrophobicity. The strains *Candida orthopsilosis* CCMA 1776, *Candida parapsilosis* CCMA 1777 and *Candida parapsilosis* CCMA1756 presented the lowest value ($P<0.05$), being then excluded from the following tests. The hydrophobic nature of the outermost surface of microorganisms is an important property that has been implicated in the attachment of the microorganism to the intestinal epithelial cells (Schillinger, Guigas, Holzapfel, 2005).

From the probiotic prospect, a high hydrophobicity of the cell surface explains why, among the yeasts, certain strains have relatively slower elimination kinetics from the gastrointestinal tract and exert different health effects (Martins et al., 2009). The Surface hydrophobicity can be used as an indirect instrument to assess a probiotic's ability to potentially adhere to the intestinal mucosa. Various authors have reported that a percentage of hydrophobicity between 30 and 40% could imply probiotics' ability to interact with mucus and at least perform transient adhesion (Abdulla, Abed, & Saeed, 2014; Ilavenil et al., 2016; Sidira, et al. 2015). All the isolates selected for the next tests showed the percentage of hydrophobicity higher than 30%, thus these isolates may have a high adhesion capacity.

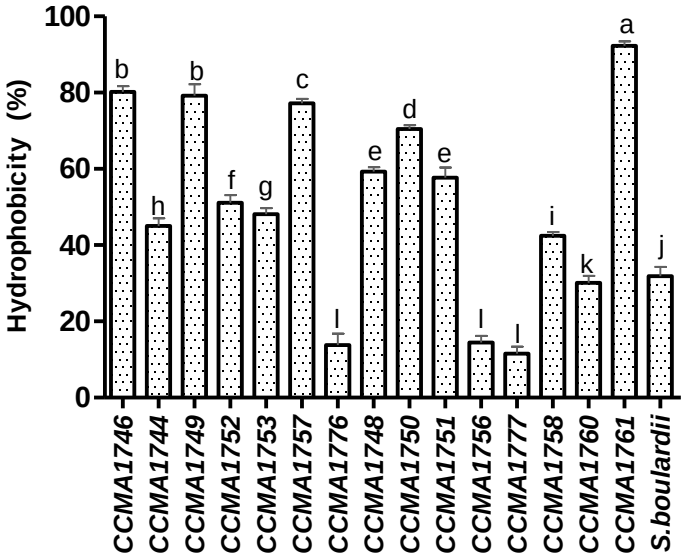

Figure 1. Percentage (%) of Hydrophobicity obtained for the different yeast isolates Bar indicate SD. Bars followed by different letters differ significantly (P< 0.05) by Scott-Knott test.

Autoaggregation assays and coaggregation assays

The ability of microorganisms to adhere to epithelial cells and mucosal surfaces is of great importance in probiotic selections as it prolongs their stay in the gut and allows them to exert their beneficial effects (Collado et al., 2008). The results of the autoaggregation assay (Table 3) indicated that all isolates evalued presented the percentage of autoaggregation between intermediate (between 30 and 60%) and high (>60%) (Kos et al. 2003) being the highest value observed for *Saccharomyces cerevisiae* CCMA1746 with a autoaggregation of

91%, similar (P>0.05) result as the one presented by the reference strain *S. boulardii*, At the end of 4 h of incubation, all isolates had a higher (P<0.05) autoaggregation percentage than that found in the first measurement time (1 h). The content of β-(1,3)-D-glucans in the cell wall (McGinnis, 2004), and some proteins in the wall surface (Singleton, et al. 2005) have been reported as the factors responsible for autoaggregation abilities of yeasts.

The variability of these results indicates that autoaggregation capacity is strongly dependent on yeast species and strain (Gil-Rodríguez et al. 2015). Strains isolated in this study were naturally grown in uncontrolled environmental conditions, as a result they showed great autoaggregation potential. This property supposedly was developed by these strains as protective mechanism in nature complex ecosystem. (Sakandar, et al. 2018). Menezes et al 2019, studying yeasts isolated Brazilian indigenous fermented food, cocoa fermentation, and kefir for the species *Pichia guilliermondii*, *Candida orthopsilosis* and *Saccharomyces cerevisiae*, the same as our study, found that the values of autoaggregation percentage ranged from 68.0 to 99,3%.

Table 3. Percentage of autoaggregation of yeast isolates

Strains	Time of incubation (hours)			
	1	2	3	4
S. cerevisiae CCMA1746	56^{bB} ±0.01	83^{bB} ±0.19	88^{aA} ±0.06	91^{aA} ±0.08
G. candidum CCMA1745	47^{cA} ±0.10	51^{cA} ±0.03	60^{cA} ±0.05	64^{bA} ±0.09
P. guilliermondii CCMA1753	25^{dA} ±0.09	38^{dA} ±0.10	44^{cA} ±0.02	41^{cA} ±0.12
C. orthopsilosis CCMA1748	35^{cB} ±0.02	70^{bA} ±0.44	48^{cA} ±0.17	51^{cA} ±0.09
C. orthopsilosis CCMA1750	57^{bA} ±0.01	63^{bA} ±0.04	68^{bA} ±0.12	74^{bA} ±0.21
C. parapsilosis CCMA1751	56^{bB} ±0.07	62^{bB} ±0.05	70^{bA} ±0.03	73^{bA} ±0.05
M. caribbica CCMA1758	45^{cA} ±0.03	44^{cA} ±0.01	47^{cA} ±0.01	54^{cA} ±0.05
G. candidum CCMA1760	41^{cA} ±0.01	47^{cA} ±0.03	53^{cA} ±0.02	53^{cA} ±0.07
D. hansenii CCMA1761	61^{bA} ±0.13	68^{bA} ±0.05	67^{bA} ±0.02	72^{bA} ±0.14
S. boulardii Floratil®	59^{bB} ±0.06	84^{aA}±0.13	84^{aA} ±0.01	87^{aA} ±0.09

Results are expressed as mean ± SD, determined in duplicate. Mean values of different letters, lowercase in columns and uppercase in rows differ significantly (P <0.05) by the Scott-Knott test

Coaggregation values ranged between 6,08 (*Candida orthopsilosis* CCMA1757) and 36.16% (*Saccharomyces boulardii* Floratil®) for the bacteria *Escherichia coli* (EPEC) CDC 055, and between 1.26% (*Candida parapsilosis* CCMA1752) and 24.66% (*Debaryomyces*

hansenii CCMA1761) for the bacteria *Salmonella* Enteritidis *S64* (Figure 2). The isolates that showed lower percentage (P<0.05) of coaggregation with both bacteria were excluded from following tests. The isolates *Debaryomyces hansenii* CCMA1761, *Meyerozyma caribbica* CCMA1758, *Candida tropicalis* CCMA1751, *Candida orthopsilosis* CCMA1750, *Pichia guilliermondii* CCMA1753, *Candida orthopsilosis* CCMA1749 and *Saccharomyces cerevisiae* CCMA1746 showed a similar (P<0.05) coaggregation values to the reference strain *S. boulardii.*

Organisms with the ability to coaggregate with other bacteria such as pathogens may have an advantage over non-coaggregating organisms, which are more easily removed from the intestinal environment (Collado et al., 2008).

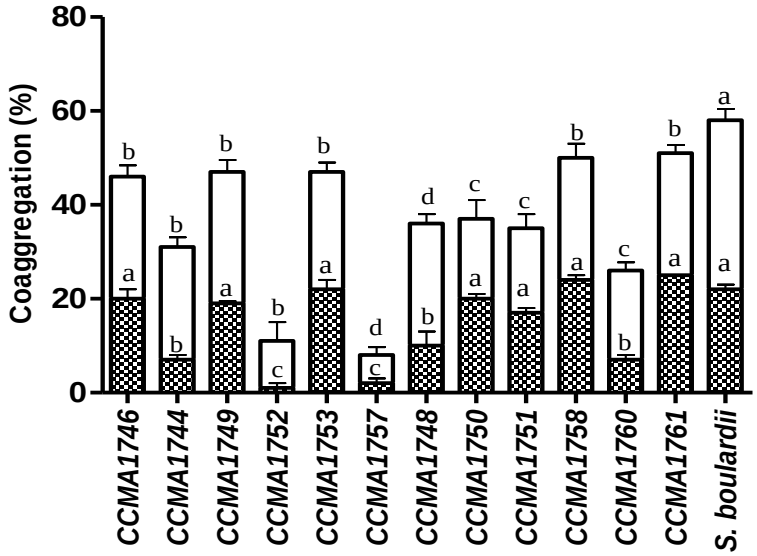

Figure 2. Percentage (%) coaggregation of yeast isolates with pathogenic microorganisms. The lower histograms represent percentage coaggregation with *Salmonella* Enteritidis S64, and the higher histograms the value the coaggregation with *Escherichia coli* (EPEC) CDC 055. Bar indicate **SD.** Bars followed by different superscript letters differ significantly (P< 0.05) by Scott-Knott test.

Antimicrobial activity

The results for antagonistic activity of the yeasts against pathogenic bacteria are shown in Table 4. The isolate *Galactomyces candidum* CCMA1744 showed no antimicrobial activity against the pathogens tested, being eliminated from the following tests. All other strains showed activity towards *Salmonella* Enteritidis ATCC 564, the strains *Pichia guilliermondii* CCMA1753, *Candida parapsilosis* CCMA1751 and *Meyerozyma caribbica* CCMA1758 showed halo of inhibitions between 20 and 30 mm (Table 4). In contrast, *S.*

aureus was inhibited by only 3 strains: *Pichia guilliermondii* CCMA1753, *Galactomyces candidum* CCMA1760 and *Saccharomyces boulardii* (Floratil®). The isolate *Candida parapsilosis* CCMA1751 had no antimicrobial activity against *Listeria monocytogenes* ATCC 19117. For the same pathogen, the isolates *Saccharomyces cerevisiae* CCMA1746, *Meyerozyma caribbica* CCMA1758 and *Galactomyces candidum* CCMA1760 had activity with halo of inhibitions greater than 20 mm.

The antimicrobial activity of yeast strains is associated to mycocins or yeast killer toxins, compounds of protein origin extracellular secreted that are mortal for some susceptible microorganism, such as yeasts, bacteria and filamentous fungi (Bussey 1972; Polonelli and Morace 1986; Izgu and Altinbay 1997; Santos et al. 2004). Killer yeast strains are immune to their own toxins, but the toxins can kill other cells by different mechanisms (Schmitt and Breinig, 2002). Several species of *Pichia* have been reported to have the killer character (Meneghin , et al 2011, Starmer, et al, 2002; Baeza, et al, 2008; Polonelli, et al, 2011), the isolate *Pichia guilliermondii* CCMA1753 from our study showed antimicrobial activity for all pathogens tested.

Table 4 Antimicrobial activity of yeast isolates

Strains	*S. aureus* ATCC 8702	*S.* Enteritidis ATCC 564	*L. monocytogenes* ATCC 19117
S. cerevisiae CCMA1746	-	++	+++
G. candidum CCMA1745	-	-	-
P. guilliermondii CCMA1753	+	+++	+
C. orthopsilosis CCMA1748	-	++	++
C.orthopsilosis CCMA1750	-	++	++
C. parapsilosis CCMA1751	-	+++	-
M. caribbica CCMA1758	-	+++	+++
G. candidum CCMA1760	+	++	+++
D. hansenii CCMA1761	-	++	++
S. boulardii Floratil®	+	++	++

Activity: + = presence of a clear zone of growth inhibition around well <10 mm; ++ = presence of a clear zone of growth inhibition around well ≥ 10 mm; +++ = presence of a clearly defined inhibition zone between 20 and 30 mm surrounding the wells containing cell-free supernatant; – = no inhibition.

Gelatinase and Hemolytic activity, DNase Production Test

None of the strains were found to be positive for gelatinase, DNase and hemolytic activity, validating their relative safety as probiotic candidates.

Survival of yeasts during in vitro digestion

Survival in the simulated gastrointestinal digestion (SGID) tract is an important criterion for determination of probiotics, and involves ability to survive the acidic gastric juice, pepsin, bile salts, basic pancreatic enzymes, and physiological temperature (Gut et al., 2018). High in vitro digestion survival for strains tested was found (Table 5). Probiotics should be supplied in quantities of 10^8 – 10^9 CFU/mL in food and that after the SGID should prevail between 10^6 and 10^7 CFU/mL to be able to exert the benefits attributed to probiotics (Shori,2017). Yeast amounts used at the beginning and those reached at the end of SGID coincide with that recommended elsewhere in the literature, all the strains maintaining viability (≥ 6.0 log10 CFU/ml) in 8th digestive step (ileum, pH 6.5, 60 min), presenting a survival rate greater than 82%.

The initial step was simulation of mouth conditions, the strains did not suffer a decrease ($P>0.05$) in viable numbers. During exposure to esophagus–stomach simulated conditions, the yeast *C. orthopsilosis* CCMA 1748 decreased ($P<0.05$) their viable cell numbers within 10 min of contact with gastric juice, which corresponds to pH 5.5. For the yeasts *S. cerevisiae* CCMA 1746 and *P. guilliermondii* CCMA 1753 the decreased ($P<0.05$) their viable cell numbers was observed after 50 min of contact with gastric juice (pH 2,8). The yeasts *M. caribiica* CCMA 1758, *C. parapsilosi* CCMA1750, *D. hansenii* CCMA 1761 maintained the concentration of viable cells significantly unchanged ($P>0.05$) until 70 min of contact in pH 2.3.

Subsequent treatment, exposition to the 7th step (duodenum, pH 5.0, 30 min) containing bile salts and pancreatin, displayed a more impact on yeasts survival. Overall, the viable cell numbers decreased ($P<0.05$). Among the six potential probiotic yeasts studied, *P. guilliermondii* CCMA 1753 and *D. hansenii* CCMA 1761 appears to be the most resistant to transit throughout the gastrointestinal tract, presenting at the end of the digestion simulation a higher ($P>0.05$) viable cell count. Similar results were also reported by Bonatsou, et al. 2015, the isolated from spontaneous fermentation of naturally black cv. Conservolea olives, *P. guilliermondii* Y16 and *D. hansenii* Y57, showed values especially high survival at simulated digestive process.

The results obtained in this work show that yeasts have ability that allow it to adapt to the stress conditions found in the gastrointestinal tract, presence of low pH, bile acids and digestive enzymes, presented high survival percentage during simulated digestion, a result which is in line with previously reported data for yeasts isolated from olive fermentation (Bonatsou, et al. 2015; Bonatsou, et al. 2018; Porru et al., 2018).

In the recent work of Romero-Luna et al, 2019, the yeast *Saccharomyces cerevisiae* Strain (C41) showed a viability of 78.95% after 180 min of exposition in of simulated digestion, result quite similar to that found in our study, where the yeast *S. cerevisiae* CCMA 1746 presented a viability of 82.08% after exposure the simulation of the gastrointestinal tract.

In general, food-associated yeasts have been found by other authors to have quite a good survival under conditions that simulate passage through the human gastrointestinal tract. (Kuhle et al., 2005; Kumura et al., 2004; Psomas et al., 2001; Trotta et al., 2012).

Table 5 Conditions used during the simulated digestion and the resultant viable cell counts of yeast isolates

Organ	Condition	Stirring (rpm)	pH	Time (min)	Viable cell counts (log CFU/mL)					
					M. caribiica CCMA 1758	*C. parapsilosi* CCMA1750	*D. hansenii* CCMA 1761	*C. orthopsilosis* CCMA 1748	*S. cerevisiae* CCMA 1746	*P. guilliermondii* CCMA 1753
Before simulation	-	-	-	-	7.16 ± 0.09^{Ac}	7.41 ± 0.01^{Aab}	7.37 ± 0.03^{Aab}	7.34 ± 0.05^{Ab}	7.42 ± 0.06^{Aab}	7.46 ± 0.01^{Aa}
Mouth	Saliva	200	6.9	2	7.16 ± 0.05^{Ae}	7.40 ± 0.08^{Aab}	7.37 ± 0.05^{Abc}	7.28 ± 0.04^{Acd}	7.41 ± 0.04^{Aab}	7.46 ± 0.03^{Aa}
Esophagus–Stomach	Pepsin	130	5.5	10	7.11 ± 0.02^{Ade}	7.38 ± 0.04^{Abc}	7.35 ± 0.02^{Ac}	7.17 ± 0.05^{Bd}	7.42 ± 0.01^{Aab}	7.47 ± 0.02^{Aa}
			4.6	10	7.10 ± 0.01^{Ac}	7.37 ± 0.02^{Aab}	7.32 ± 0.08^{Ab}	7.08 ± 0.01^{BCc}	7.44 ± 0.08^{Aa}	7.45 ± 0.04^{Aa}
			3.8	10	7.06 ± 0.09^{Ab}	7.34 ± 0.01^{Aa}	7.35 ± 0.05^{Aa}	7.07 ± 0.09^{Cb}	7.33 ± 0.05^{ABa}	7.42 ± 0.03^{Aa}
			2.8	20	7.05 ± 0.04^{Acd}	7.32 ± 0.09^{Aa}	7.34 ± 0.01^{Aa}	7.00 ± 0.02^{CDde}	7.23 ± 0.03^{Bb}	7.08 ± 0.07^{Bc}
			2.3	20	6.92 ± 0.05^{Bbc}	7.00 ± 0.07^{Bbc}	7.29 ± 0.08^{Aa}	6.91 ± 0.04^{Dc}	7.03 ± 0.04^{Cb}	6.64 ± 0.02^{Cd}
			2.0	20	6.34 ± 0.06^{Cc}	6.98 ± 0.04^{BCa}	7.07 ± 0.01^{Ba}	6.27 ± 0.09^{Ec}	6.51 ± 0.04^{Db}	6.52 ± 0.03^{Db}
Duodenum	Pancreatin + bile salt	45	5.0	30	6.16 ± 0.08^{Dd}	6.89 ± 0.04^{Ca}	6.49 ± 0.04^{Cb}	6.29 ± 0.01^{Ec}	6.05 ± 0.09^{Ede}	6.37 ± 0.08^{Ec}
Ileum	-	45	6.5	60	6.15 ± 0.06^{Db}	6.12 ± 0.09^{Dbc}	6.34 ± 0.08^{Da}	6.03 ± 0.01^{Fc}	6.09 ± 0.08^{Ebc}	6.36 ± 0.05^{Ea}
Survival (%)					85.89	82.59	86.02	82.15	82.08	85.25

Steps 1: stages to simulate the conditions in the mouth; Steps 2 to 7: stages to simulate the conditions in the esophagus - stomach; step 8: stage to simulate the conditions in the duodenum; step 9: stage to simulate the conditions in the ileum. ± indicates standard deviations from the mean. Mean values followed by different letters, uppercase in columns and lowercase in rows differ significantly ($P < 0.05$) by the Tukey's test. Survival at simulated digestion (%) = (Final viable count (log10 CFU/ml) / Initial viable count (log10 CFU/ml)) x 100

Caco-2 and HT-29 cell line adhesion assay

In the previous sections, we performed the characterization of essential factors to evaluate the probiotic potential of olive isolated yeasts. In addition, an important criterion for the selection of probiotic strains is the ability to temporarily colonize the intestine by adhering to the intestinal epithelium. For in vitro evaluation, human colon tumor cell lines such as Caco-2 and HT-29 have been used to elucidate the mechanisms involved in interactions between the microorganism and the intestinal mucosa of the host (Gheziel et al., 2018).

In the assay using the Caco-2 strain, most isolates had a high adhesion rate, with values above 50% (Figure 3). Our findings are similar to other studies that found hight adhesion capacity of isolated food yeasts (Kumura et al., 2004; Kourelis, et al., 2010; Živković et al., 2015; Menezes et al., 2019). The results are expressive compared to the study of Bonatsou et al. (2018), in which yeasts isolated from the natural fermentation of black olives showed adhesion rates ranging from 1 to 6%. The highest Caco-2 adhesion values were obtained by *Meyerozyma caribbica* CCMA 1758 (82%), followed by *Debaryomyces hansenii* CCMA 1761 (76%), *Pichia guilliermondii* CCMA1753 (72%) and *Candida orthopsilosis* CCMA1748 (71%), this adherence rates were greater ($P<0.05$) than control yeast *Saccharomyces boulardii*. For strain *Galactomyces candidum* CCMA 1760, it was observed a lower ($P<0.05$) adhesion than the reference strain, so this strain was excluded from the study. The other evaluated yeasts showed similar ($P>0.05$) values to control yeast.

Subsequently, the adhesion assay was performed on HT-29 cells. (Figure 3). This cell line differs from Caco-2 as it produces mucus, a protective barrier to the intestinal mucosa (Ringot-Destrez et al., 2018). The adhesion assay with the HT-29 cell line showed satisfactory results, with values also above 50%. The highest ($P<0.05$) percentage was observed for *Candida orthopsilosis* CCMA 1748 with a value of 87%, being greater than the reference strain yeast. It is possible that this result may be associated with the physical entrapment of microbial cells in the mucus rather than the greater specific affinity of this line for strains (Gopal et al., 2001). In the recent study by Hong et al. (2019), the values obtained for two strains of *S. cerevisiae* were lower compared to our studies, with the results ranging between 8 and 21%. *Meyerozyma caribbica* CCMA 1758 and *Pichia guilliermondii* CCMA 1753 showed values similar ($P>0.05$) to *S. boulardii*. *Candida parapsilosis* CCMA1750 isolate had the lowest adhesion capacity ($P<0.05$) of yeast strains to HT-29 cells, so it was not selected for the next test.

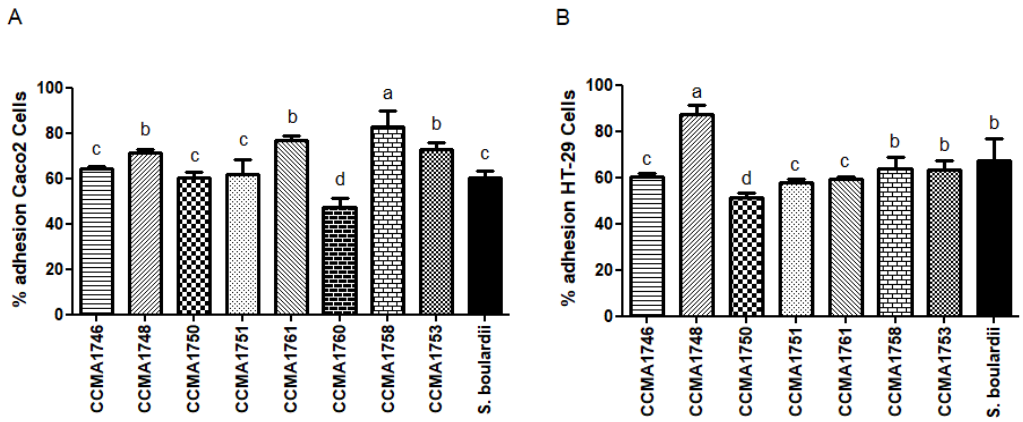

Figure 3. Adhesion capacity of yeast strains to Caco-2 (A) and HT-29 (B) cells. Bar indicate SD. Bars followed by different letters differ significantly ($P < 0.05$) by Scott-Knott test.

Inhibition of pathogenic bacteria adhesion to Caco-2 by yeasts

An important aspect of the function of probiotic microorganisms is the protection of the host's gastrointestinal tract against invasive pathogens. The resident GIT microbiota is believed to provide host protection against possible colonization by enteropathogenic microorganisms (Reid et al., 1990). Probiotic microorganism may have important properties to inhibiti of pathogen adhesion to host cells, including competition for receptors on the target cell, production of a toxic environment, production of molecules with antimicrobial activity and coaggregation with pathogens (Oelschlaeger, 2010).

In this study, it was evaluated the ability of the seven selected yeasts to inhibit the adhesion of *Salmonella* Enteritidis S64 (Table 6) and *Escherichia coli* CDC 055 (Table 7) pathogens to the Caco-2 cell, in exclusion and competition assays.

Usually, yeast inoculation substantially reduced pathogen adhesion to the epithelial cell surface, in some cases being more efficient than reference strain, *S. boulardii*. For *Salmonella* Enteritidis, the strain *Candida orthopsilosis* CCMA1748 showed the higher ($P<0.05$) percentage of inhibition in the competition assay, higher than the reference strain. For the exclusion assay the strains *Candida tropicalis* CCMA1751, *Saccharomyces cerevisiae* CCMA1746 and *Candida orthopsilosis* CCMA1748, presented higher ($P<0.05$) inhibition percentage than the reference strain.

For the pathogen *E. coli*, all the strains showed similar ($P>0.05$) adhesion inhibition capacity to the reference strains in the competition assay. The strain *Candida tropicalis* CCMA1751 presented results similar ($P>0.05$) to those found for the reference strain for the

exclusion assay, and the other isolates showed the higher (P<0.05) adhesion inhibition capacity than the *S. boulardii*.

In general, the inhibition of both pathogens was higher (P<0.05) for the exclusion assays when the yeast was previously inoculated compared to the concurrent inoculation in competition assay. This is associated with the ability of yeast to adhere to the intestinal mucosa, preventing pathogens from adhering through a physical barrier (Cardarelli et al., 2003).

The interaction between pathogens and intestinal epithelium occurs through the interaction of receptors and cellular structures, and each entero-invasive bacterium uses different pathways for cell invasion (Burkholder, Bhunia, 2009; Nakamura et al., 2012; Zeiner, 2012). A study suggests that *Salmonella Typhimurium* adhesion to Caco2 cells is mediated by type 1 bacterial fimbriae (Burkholder, Bhunia, 2009). The presence of this type of fimbria increases coaggregation with *S. boulardii*, due to its affinity for mannose, common in yeast cell wall composition (Gedek, 1999). Thus, we believe that the lower adhesion of pathogens to intestinal cells promoted by the tested yeasts can occur through the trapping of pathogenic bacteria on its surface.

Table 6 Percentage inhibition of *S.* Enteritidis S64 adhesion to Caco-2 cells by yeasts

Strains	Competition (%)	Exclusion (%)
S. cerevisiae CCMA 1746	19.45 ^{bB}±4.70	60.21^{aA}±5.64
P. guilliermondii CCMA 1753	15.76 ^{bA}±8.00	33.76 ^{bA}±4.94
C. orthopsilosis CCMA 1748	60.40 ^{aA}±2.34	67.90 ^{aA}±4.34
C. tropicalis CCMA 1751	32.45 ^{bB}±1.24	69.09 ^{aA} ±0.00
M. caribbica CCMA 1758	21.91 ^{bB} ±3.97	44.87 ^{bA} ±1.17
D. hansenii CCMA 1761	33.88 ^{bA}±5.08	46.85^{bA}±1.85
S. boulardii Floratil®	26.84 ^{bA}±2.98	37.02 ^{bA} ±10.37

Displayed Log CFU / ml values were determined in duplicate; ± indicates standard deviations from the mean. Mean values followed by different letters, lowercase in columns and uppercase in rows differ significantly (P <0.05) by the Scott Knott test.

Table 7 Percentage inhibition of E. coli CDC 055 adhesion to Caco-2 cells by yeasts

Strains	Competition (%)	Exclusion (%)
S. cerevisiae CCMA 1746	22.47 ^{aB}±1.63	40.95 ^{aA}±1.15
P. guilliermondii CCMA 1753	23.43 ^{aB}±0.84	38.55 ^{aA}±3.48
C. orthopsilosis CCMA 1748	23.55^{aB}±2.78	38.01 ^{aA}±2.33
C. tropicalis CCMA 1751	21.82 ^{aA} ±1.32	23.86 ^{bA} ±2.13
M. caribbica CCMA 1758	21.79^{aB} ±0.79	37.24 ^{aA} ±1.4
D. hansenii CCMA 1761	20.37^{aB}±0.85	37.99 ^{aA}±0.57
S. boulardii Floratil®	23.64 ^{aA} ±1.48	24.16 ^{bA} ±1.92

Displayed Log CFU / ml values were determined in duplicate; ± indicates standard deviations from the mean. Mean values followed by different letters, lowercase in columns and uppercase in rows differ significantly (P <0.05) by the Scott Knott test.

Conclusion

In the fermentation of Brazilian table olives, a product little explored in relation to the microbiota, yeast strains with potential probiotic properties can be selected. From a total of 18 yeasts, 6 showed potential properties for use as probiotics: *Saccharomyces cerevisiae* CCMA1746, *Pichia guilliermondii* CCMA1753, *Candida orthopsilosis* CCMA1748, *Candida tropicalis* CCMA1751, *Meyerozyma caribbica* CCMA1758, and *Debaryomyces hansenii* CCMA1761. The results obtained show that yeasts have ability that allow it to adapt to the stress conditions like a presence of low pH, bile acids and digestive enzymes, so being able to reach and remain viable in the gastrointestinal tract and provide the beneficial effect. In addition, the yeast strains exhibited antimicrobial activity against pathogens. Analyses of surface properties, the isolates showed autoaggregation capacity, coaggregation with enteropathogens, showed high adhesion values to Caco-2 and HT-29 cells, and were also able to inhibit pathogen adhesion.

The study makes a relevant discovery for the characterization of new potential probiotic strains, mainly yeasts that are still little explored when compared with bacteria. The exploration of national fermented products is important to find species with probiotic potential associated, can be used for the development of new foods and probiotic products. Further *in vivo* studies should be also performed to confirm its potential beneficial effects

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Compliance with Ethical Standards

Conflicts of Interest: The authors declare that they have no conflict of interest.

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