ALINE GALVÃO TAVARES MENEZES

CHARACTERIZATION OF YEAST WITH PROBIOTIC POTENTIAL AND ITS USE IN FERMENTED NON-DAIRY BEVERAGE

LAVRAS – MG

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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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APROVADA em 09 de agosto de 2018.

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2018

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-Chico Xavier-

RESUMO

O estudo de características probióticas de diferentes microrganismos e novos alimentos os contendo tem se tornado de grande importância, as leveduras tem demonstrado apresentar características importantes para serem utilizadas como probióticas. O presente trabalho teve como objetivo selecionar leveduras potencialmente probióticas isoladas de alimentos fermentados brasileiros, como kefir, cacau e bebidas indígenas e desenvolver uma bebida fermentada não láctea. Um total de 116 leveduras foram caracterizadas *in vitro* quanto aos atributos probióticos. Trinta e seis foram tolerantes a condições gastrointestinais avaliadas pela tolerância ao pH 2,0, sais biliares (0,3% m/v) e temperatura de 37 °C. Destes, 15 isolados apresentaram percentual semelhante ou maior (P <0,05) de hidrofobicidade, autoagregação e coagregação com *Escherichia coli,* que a linhagem probiótica comercial S*accharomyces boulardii*. Todas essas cepas apresentaram alto percentual de adesão às células Caco-2 (> 63%) e atividade antioxidante (variando de 18 a 62%). Também foi avaliada a capacidade das leveduras em inibir a adesão e infecção dos patógenos alimentares *E. coli*, *Salmonella* Enteritidis e *Listeria monocytogenes* em células Caco-2. As cepas *S. cereviseae* CCMA 0731, *S. cereviseae* CCMA 0732 e *Pichia kluyveri* CCMA 0615 apresentam co-agregação e considerável redução da infecção por patógenos em células Caco-2, sendo iguais ou maiores que a cepa comercial S*accharomyces boulardii*. Para o desenvolvimento de uma bebida à base de milho, a cepa comercial probiótica *Lactobacillus paracasei* LBC-81 foi testada em combinação com as leveduras potencialmente probióticas *S. cereviseae* CCMA 0731, *S. cereviseae* CCMA 0732 e *Pichia kluyveri* CCMA 0615. Todas as cepas testadas apresentaram viabilidade superior a 6 log UFC/mL, exceto *P. kluyveri*. Os ácidos lático e acético foram os principais ácidos orgânicos produzidos durante a fermentação. O etanol foi detectado nos ensaios com *S. cerevisiae*; porém em concentrações inferiores a 0,5 g/L, caracterizando uma bebida não alcoólica. Setenta compostos voláteis foram detectados, incluindo ácidos, álcoois, aldeídos, ésteres, cetonas e outros compostos. A análise sensorial mostrou que os consumidores nem gostaram e nem desgostaram, o que é um resultado interessante já que não foram adicionados flavorizantes e/ou adoçantes. Três leveduras potencialmente probióticas foram selecionadas através de um screening e caracterizadas neste trabalho, estas foram utilizadas na produção de bebida fermentada a base de milho, caracterizando um alimento probiótico não lácteo, alternativo para o mercado consumidor cada vez mais exigente.

Palavras-chave: Probiótico, bebidas não lácteas, bebida probiótica, *Saccharomyces cerevisiae*

ABSTRACT

The study of probiotic characteristics of different microorganisms and novel foods containing them has become of great importance. Specifically, it has been shown that yeasts have valuable probiotic characteristics. This work aimed to select potentially probiotic yeasts isolated from Brazilian fermented foods such as kefir, cocoaand indigenous beverages and to develop a non-dairy fermented beverage. *In vitro* characterization of probiotic traits was performed on 116 yeasts. Thirty-six yeasts were tolerant to gastrointestinal conditions evaluated by tolerance to pH 2.0, bile salts (0.3% m/v) and temperature of 37 °C. Out of the 36 yeasts, 15 isolates had the percentage of hydrophobicity, autoaggregation and co-aggregation with *Escherichia coli* similar or higher (P <0,05) than the commercial probiotic strain *Saccharomyces boulardii*. All these strains had high percentage of adhesion to Caco-2 cells (> 63%) and antioxidant activity (ranging from 18 to 62%). The ability of yeasts to inhibit adhesion and infection of the food pathogens *E. coli*, *Salmonella* Enteritidis and *Listeria monocytogenes* in Caco-2 cells was also evaluated. The strains *S. cereviseae* CCMA 0731, *S. cereviseae* CCMA 0732 and *Pichia kluyveri* CCMA 0615 showed co-aggregation and considerable reduction of infection by pathogens in Caco-2 cells, being equal or greater than the commercial strain *Saccharomyces boulardii*. For the development of a corn-based beverage, the commercial probiotic strain *Lactobacillus paracasei* LBC-81 was tested in combination with the potentially probiotic yeasts *S. cereviseae* CCMA 0731, *S. cereviseae* CCMA 0732 and *Pichia kluyveri* CCMA 0615. All tested strains showed viability higher than 6 log CFU/mL, except *P. kluyveri*. Lactic and acetic acids were the main organic acids produced during fermentation. Ethanol was detected in the *S. cerevisiae* assays, but at concentrations lower than 0.5 g/L, characterizing a non-alcoholic beverage. Seventy volatile compounds have been detected, including acids, alcohols, aldehydes, esters, ketones and other compounds. Sensory analysis showed that consumers neither liked nor disliked it, which is an interesting result since no flavoring and/or sweetening agents were added. Three potentially probiotic yeasts were selected through a screening and characterized in this work. These yeasts were used in the production of corn-based fermented beverage, characterizing a non-dairy probiotic food, which is an alternative to the increasingly demanding consumer market.

Keywords: Probiotics, non-dairy beverages, probiotic beverage, *Saccharomyces cerevisiae*

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PRIMEIRA PARTE

1 INTRODUÇÃO

O termo probiótico é usado para definir microrganismos vivos utilizados como alimentos ou suplementos farmacêuticos que beneficiam a saúde humana e animal quando ingeridos, através da melhoria do balanço da microbiota do intestino (ARAGON-ALEGRO et al., 2007; MARTINS et al., 2005). Critérios para seleção de probióticos incluem tolerância às condições gastrointestinais (ácidos e sais biliares), não serem patogênicos, capacidade de aderir à mucosa intestinal e a exclusão competitiva de patógenos (COLLADO, MERILUOTO E SALMINEN 2008), estes podem ser utilizados e encontrados em alimentos fermentados.

Nos últimos anos, um grande número de novos produtos foi lançado no mercado alimentício e farmacêutico, atraindo a atenção e interesse dos consumidores por seu apelo aos benefícios à saúde. A população está mais consciente e preocupada em relação à alimentação saudável, assim, a indústria tem buscado novas alternativas para o desenvolvimento de alimentos que são capazes de promover a saúde, como por exemplo, os probióticos.

Alimentos probióticos são alimentos que contém uma cultura única ou mista de microrganismos que vão trazer benefícios à saúde do consumidor, melhorando o equilíbrio microbiano no sistema gastrointestinal (FULLER, 1989). A maioria dos probióticos atualmente comercializados é de origem bacteriana, espécies de lactobacilos e bifidobactérias principalmente, a maioria das leveduras é sensível às condições do trato gastrintestinal, devido a esse fator, poucas leveduras são conhecidas comercialmente. *Saccharomyces cerevisiae* var. *boulardii* é a única reconhecida e caracterizada como probiótica (CZERUCKA; PICHE; RAMPAL, 2007; HILL et al., 2014).

Tradicionalmente os probióticos são adicionados a produtos lácteos fermentados, porém, hoje em dia há um aumento da demanda dos consumidores por produtos probióticos alternativos, como por exemplo, produtos não lácteos ou suplementos alimentares comercializados em comprimidos e cápsulas (RIVERA-ESPINOZA; GALLARDO-NAVARRO, 2010).

Alimentos fermentados espontaneamente podem constituir um reservatório para novas estirpes de leveduras com potenciais características probióticas. Vários produtos fermentados espontaneamente produzidos no Brasil podem ser fontes de leveduras e bactérias, estes incluem as bebidas fermentadas indígenas como caxiri e cauim, o kefir e a fermentação de

cacau para produção de chocolate. Estes compreendem uma mistura simbiótica microbiana complexa (DE MELO PEREIRA; MAGALHÃES-GUEDES; SCHWAN, 2013; GABRIELA et al., 2017; MOREIRA et al., 2013b)

O processo de fermentação é importante para indústria, pois prolonga a vida útil, melhora o valor nutricional e reduz o risco de toxinfecções. Além disso, tanto os microrganismos procariotos como os eucariotos que estão envolvidos no processo fermentativo podem ter efeitos benéficos sobre a saúde humana e o bem-estar (PEDERSEN et al., 2012).

Como o mercado de produtos probiótico cresce continuamente, a investigação sobre o desenvolvimento de novos produtos fermentados não lácteos contendo microrganismos probióticos e o isolamento de novas estirpes com potenciais propriedades probióticas é de grande importância.

2 REFERENCIAL TEÓRICO

2.1 Probióticos

A palavra "probiótico" é derivada da linguagem grega e significa "para a vida" (FULLER, 1989). De acordo com a definição da Food and Agriculture Organization, probióticos são definidos como “microrganismos vivos que, quando administrados em quantidades adequadas, conferem benefícios à saúde do hospedeiro” (FAO, 2001).

O conceito probióticos não é recente, há muitos anos eles já eram consumidos pelos seres humanos na forma de alimentos fermentados (CZERUCKA; PICHE; RAMPAL, 2007; RANADHEERA; BAINES; ADAMS, 2010). No início do século 20, o imunologista russo Elie Metchnikoff sugeriu que os lactobacilos ingeridos em iogurte teriam influência positiva sobre a microbiota do intestino (METCHNIKOFF, 1907). Muitos cientistas, tais como Hipócrates e outros receitavam leites fermentados para distúrbios de estômago e intestino (LOURENS-HATTINGH; VILJOEN, 2001). Hoje é aceito que os probióticos contribuem com equilíbrio da microbiota intestinal e evita distúrbios gastrointestinais, quando consumidos regularmente (LAVERMICOCCA, 2006).

Os probióticos podem ser constituídos de bactérias ou leveduras, porém a maioria são bactérias, principalmente bactérias do ácido lático, são as mais utilizadas no preparo de probióticos (CZERUCKA; PICHE; RAMPAL, 2007).

Alguns exemplos de espécies de microrganismos que têm sido utilizados como probióticos ao longo dos anos são *Lactobacillus bulgaricus*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus fermentum*, *Lactobacillus paracasei*, *Lactobacillus reuteri*, *Lactobacillus rhamnous, Lactobacillus helveticus*, *Lactobacillus lactis*, *Streptococcus thermophilus*, *Enterococcus faecium*, *Enterococcus faecalis*, *Escherichia coli,* espécies de *Bifidobacterium*, *Bacillus,* e a levedura *Saccharomyces boulardii* (RANADHEERA et al., 2010; SINGH et al., 2011). Porém existem preocupações em torno da segurança do uso de espécies de *Escherichia*, *Bacillus* e *Enterococcus* como probióticos, já que esses gêneros contêm espécies patogênicas oportunistas (MARTINS et al., 2009).

O uso de microrganismos probióticos, ocorre principalmente em alimentos lácteos, como: iogurtes, kefir e bebidas fermentadas. Os produtos lácteos são principal fonte carregadora de probióticos, provavelmente devido ao leite ajudar a tolerar as condições severas do trato gastrointestinais, já que sua ação tampão pode proteger os probióticos em tais condições, reduzindo sua exposição direta a condições adversas (RANADHEERA et al., 2017).

O quadro 1 mostra alguns exemplos de produtos probióticos disponíveis no mercado e seus fabricantes.

Tabela 1- Exemplos de probióticos disponíveis no mercado.

Cepa	Nome comercial	Fabricante
Bifidobacterium animalis DN 173 010	Activia	Danone
Lactobacillus casei DN-114 001	Actimel	Danone
L. johnsonii La1 (Lj1)	LC1	Nestlé
L. casei Shirota	Yakult	Yakult
Saccharomyces boulardii	DiarSafe,Floratil	Biocodex, Merck
L. casei Immunitas	DanActive	Dannon
Bifidobacterium lactis HN019	Chocolate probiotic plus D	Biorela
Lb. rhamnosus, Lb. Reuteri	RepHresh Pro-B	RepHresh
L. johnsonii, L. helveticus	Chamyto	Nestlé
B. lactis, L acidophilus	Dietalact	Parmalat
B. lactis, L. acidophilus	Iogurte Biofibras	Batavo
L.casei, B.lactis, L. acidophilus	Leite Fermentado Parmalat	Parmalat
L. casei	Batavito	Batavo

Fonte: Adaptado de Guarner et al., 2008.

O mercado de produtos probióticos é bem promissor. A influência dos hábitos e necessidades dos consumidores estimula o desenvolvimento das indústrias, aumentando assim, a competitividade por novos produtos a preços acessíveis. De acordo com a Euromonitor Internatinal, o mercado global de probióticos tem um valor de cerca de US$ 43 bilhões, o iogurte continua sendo o modo dominante de comercialização, e os suplementos probióticos são os produtos de crescimento mais rápido. Os iogurtes são responsáveis por 76% das vendas de probióticos, produtos de leite fermentado com 14%, enquanto os suplementos probióticos detém 10% do mercado. O mercado global de probióticos em 2013 foi estimado em US$ 32,06 bilhões e deverá atingir US$ 52,34 bilhões até 2020.

Quando ingeridos oralmente, os microrganismos probióticos devem sobreviver a condições adversas durante a sua passagem através do trato gastrointestinal (TGI) e ser capaz de influenciar a microbiota do intestino humano. Eles podem desempenhar um importante papel ajudando o corpo a se proteger contra infecção, especialmente colonizando a superfície da mucosa do trato gastrointestinal. Devem estar presentes como células viáveis de preferência em grande número (ARAGON-ALEGRO et al., 2007; SINGH et al., 2011; PERRICONE et al., 2014).

A dosagem do probiótico varia dependendo do produto e da sua indicação. Não existe consenso sobre o número mínimo de microrganismos que devem ser ingeridos para obter um efeito. Um probiótico deve conter vários bilhões de microrganismos para aumentar assim a probabilidade de colonização adequada do intestino. A indústria de alimentos em geral, tem adotado o nível mínimo recomendado de 10^6 UFC/mL para leites fermentados inoculados com bifidobactérias no momento de consumo. A *Food and Agriculture Organization* recomenda 10^9 células viáveis ou 10^7 células viáveis/100mL como sendo a dosagem diária recomendada para se obter os efeitos benéficos através de um alimento probiótico (FAO, 2001). Deve-se levar em consideração o efeito do armazenamento na viabilidade do probiótico (WILLIAMS, 2010).

Para lactobacilos, em geral, utiliza-se doses de 1 a 2 x 10^7 UFC por dia. Para *S. boulardii*, a maioria dos estudos recomendam de 250 a 500 mg do liofilizado (WILLIAMS, 2010). No entanto, a dosagem pode variar de acordo com produto a ser utilizado, como por exemplo, o floratil®, em que cada cápsula contém 200 mg de *Saccharomyces boulardii* liofilizada, sendo que 200 mg de liofilizado contêm no mínimo 1 x 10^9 células (MCFARLAND, 2010).

Durante o desenvolvimento de alimentos probióticos, culturas probióticas são artificialmente introduzidas no alimento. A maior parte das preparações de culturas está comercialmente disponível em altas concentrações, em forma congelada ou liofilizada. Culturas congeladas contêm mais que 10^{10} UFC/g, ao passo que as culturas liofilizadas normalmente apresentam mais que 10^{11} UFC/g (TRIPATHI; GIRI, 2014).

Segundo Collado et al. (2007), cada microrganismo apresenta características próprias, sendo assim, produto preparado com combinação de cepas probióticas pode aumentar os benefícios sobre o hospedeiro quando comparado com uso de uma única cepa, isso porque ocorre efeito sinérgico.

2.2 Seleção de microrganismos probióticos

Alguns critérios para a seleção de novas cepas probióticas incluem características relativas à funcionalidade, segurança e aos aspectos tecnológicos (SAARELA et al., 2000).

Para que um microrganismo seja caracterizado como probiótico e exerça seu papel fisiológico no intestino, eles devem ser capazes de lidar com algumas barreiras antes de atingirem o local alvo. A passagem pelo trato gastrintestinal possui fases de estresse que podem afetar a viabilidade celular desses microrganismos, a principal é o pH ácido do estômago, resultado do suco gástrico, além da resistência aos sais biliares presente no duodeno e a enzimas digestivas. A capacidade dos microrganismos em sobreviver ao estômago é um fator importante para sua seleção. Após a passagem por essas barreiras, os microrganismos ainda devem ser capazes de multiplicação e colonização do intestino (FERNANDEZ-PACHECO et al., 2018; RODRÍGUEZ et al., 2018; SAARELA et al., 2000).

A fim de manifestar efeitos benéficos, a capacidade dos probióticos em se agregar, formar agregados entre si, torna-se uma propriedade desejável. Os microrganismos com a capacidade de co-agregação com outros microrganismos, ou seja, formar agregados, por exemplo, com bactérias patogénicas, pode ter grandes vantagens sobre microrganismos que não possuem essa capacidade, pois assim, podem eliminá-las juntamente com as fezes, além disso, a habilidade de formar agregados, forma uma barreira que impede a colonização de bactérias patogénicas (COLLADO, MERILUOTO E SALMINEN 2007).

Os modos de ação pelos quais os probióticos contribuem para a saúde humana enquadram-se basicamente em três principais categorias: primeiro, os probióticos podem excluir ou inibir microrganismos patogênicos, sendo atualmente o mecanismo mais estudado.

O segundo é referente à melhorar a função da barreira epitelial intestinal através da indução da produção de muco e de mecanismos de defesa, e o terceiro método, é a modulação da resposta imune do hospedeiro (LEBEER; VANDERLEYDEN; DE KEERSMAECKER, 2010).

Os microrganismos probióticos atuam também como barreira física de defesa no intestino, impedindo que os microrganismos patogênicos possam exercer seus efeitos e reduzindo a área de adesão destes (SAAD, 2006). A atividade antipatogênica é considerada um dos efeitos mais benéficos dos probióticos, ao contrário dos antibióticos clássicos, com uso de probióticos, não ocorre alteração na composição da população microbiana original (KERRY et al., 2018).

A aderência de microrganismos envolve vários fatores, segundo Kos et al., 2003 essa ligação é baseada em interações físico-químicas, que estão relacionadas às cargas elétricas presentes e a hidrofobicidade da parede celular do microrganismo.

Além disso, estes não devem ser tóxicos nem patogênicos, causar alergias ou serem mutagênicos (NAGPAL et al., 2012). Seu uso não deve interferir na aceitação do alimento funcional, quando adicionados a alimentos, devem ser facilmente cultiváveis em grande escala, ter resistência a manipulações tecnológicas e sobreviver ao processamento dos alimentos, como aquecimento e baixas condições de oxigênio (DALIRI; LEE, 2015; NAGPAL et al., 2012). Deve também ser observada a habilidade da cultura em coexistir com a microbiota do hospedeiro (FAO/WHO, 2002).

2.3 Microbiota do trato gastrointestinal

O trato gastrointestinal (TGI) animal é dividido em regiões anatômicas bem definidas que inclui: estômago, intestino delgado (duodeno, jejuno e íleo) e intestino grosso (cólon e ceco). Sua função é a de converter os alimentos em componentes que podem ser digeridos e absorvidos pelo hospedeiro (BARROSO et al., 2015; LEE et al., 2015; ZOETENDAL et al., 2004).

O TGI está em constante exposição a microrganismos do ambiente externo, devido a isso, ele possui sistemas de proteção, que inclui: o baixo pH do estômago, a cobertura completa do trato com uma camada de muco, e a presença de microrganismos que colonizam o trato (ZOETENDAL; RAJILIC-STOJANOVIC; DE VOS, 2008). O intestino humano

adulto possui um alto número de microrganismos, o tamanho da população é de até 100 trilhões de microrganismos (BÄCKHED et al., 2005).

A composição da microbiota do TGI não é homogênea, o intestino delgado abriga apenas algumas espécies e um número reduzido de microrganismos. As populações bacterianas aumentam no TGI, atingindo números mais altos e maior diversidade no cólon (SEKIROV et al. 2010).

Porém, comunidades microbianas se diferem entre os indivíduos, de acordo com a idade, alimentação e condições de saúde. Apesar das diferenças entre os indivíduos, há indícios de que alguns microrganismos são compartilhados por pessoas diferentes (ZOETENDAL; RAJILIC-STOJANOVIC; DE VOS, 2008).

Os primeiros estudos sobre a microbiota intestinal humana, com base na cultura de organismos obtidos a partir de matéria fecal, revelou a existência dos três domínios da vida, Bacteria, Archaea e Eukarya (BÄCKHED et al., 2005; RAJILIĆ-STOJANOVIĆ; SMIDT; DE VOS, 2007). Sendo a maioria das bactérias presentes no TGI dos filos Bacteroides e Firmicutes, proteobactérias são comuns, mas geralmente não são dominantes (BÄCKHED et al., 2005). Contando que cerca de 80% da microbiota intestinal não pode ser cultivada sob condições laboratoriais padrão (ECKBURG et al. 2005) e com o avanço na utilização de métodos independentes de cultura para a projeção microbiana, obtiveram-se progressos, sabe-se agora que os filos predominantes no estômago são Actinobacteria, Firmicutes, Bacteroidetes e Proteobacteria, enquanto que no cólon, são frequentemente encontrados os filos Proteobacteria, Firmicutes e Bacteroidetes (MENG et al., 2018).

Bacteria é o grupo de microrganismos mais estudado, no entanto, os procariotos não são os únicos componentes da microbiota intestinal dos mamíferos. Os papéis dos eucariotos presentes no intestino dos seres humanos estão apenas começando a ser investigado (HALLEN-ADAMS et al., 2015). As leveduras compõem menos do que 0,1% da microbiota do intestino. A maioria dos isolados de leveduras a partir do TGI são *Candida albicans*, *Torulopsis glabratra* e *Candida tropicalis*. Embora as leveduras sejam uma minoria dos organismos que compõem a microbiota, o seu tamanho de célula é 10 vezes maior que das bactérias, o que pode ser significativo para serem utilizadas como probióticas (CZERUCKA; PICHE; RAMPAL, 2007).

A microbiota presente no TGI exerce diversas funções importantes na saúde humana, incluindo a manutenção da homeostase intestinal, o TGI é um ecossistema extremamente complexo que coexiste em equilíbrio com o hospedeiro, este oferece um ambiente rico em

nutrientes a microbiota, que por sua vez, exerce efeitos benéficos ao hospedeiro, quando este equilíbrio é perturbado, ocorrem distúrbios clínicos (CZERUCKA; PICHE; RAMPAL, 2007; HOOPER; LITTMAN; MACPHERSON, 2012).

2.4 Probióticos e benefícios à saúde

A microbiota intestinal desempenha um importante papel na saúde do hospedeiro, influenciando seu sistema imunológico a absorção de nutrientes e regulando o metabolismo energético (MARQUES, et al., 2014).

Existem várias evidências que apoiam potenciais aplicações clínicas dos probióticos na prevenção e tratamento de doenças, eles podem fornecer uma série de benefícios para a saúde, principalmente, através da manutenção de um bom equilíbrio e composição da microbiota intestinal, ajudando a aumentar a capacidade do organismo para resistir à invasão de agentes patogênicos e manter o bem-estar do hospedeiro (RANADHEERA; BAINES; ADAMS, 2010).

Um alvo comum dos probióticos tem sido associado com o TGI, principalmente devido a sua capacidade em restaurar a microbiota intestinal (WILLIAMS, 2010).

Os probióticos têm sido amplamente utilizados em aplicações terapêuticas, incluindo a prevenção de doenças urogenitais, como vaginite ocasionada por *Candida*. A composição microbiana urogenital é influenciada pela genética, pelos níveis de hormônio, pela higiene, uso de contraceptivos, antibióticos, entre outros fatores. O desequilíbrio do ecossistema urogenital feminino pode ser causado por diversos fatores externos ou internos, afetando negativamente a microbiota indígena e favorecendo o crescimento excessivo de agentes potencialmente patogênicos (SAMNALIEV; LIGHTDALE, 2011). Os probióticos afetam a microbiota vaginal restaurando o equilíbrio do ecossistema pela colonização, ocorrendo um aumento das populações de microrganismos benéficos e uma diminuição dos patogênicos (NADER-MACÍAS; SILVINA; TOMÁS, 2015).

Everard et al. (2014), demonstraram que a administração de *S. boulardii* em ratos obesos e diabéticos tipo 2, ocorriam modificações no metabolismo do hospedeiro e estava associada a alterações na composição microbiana do intestino. Os camundongos tratados com *S. boulardii* apresentaram diminuição da massa gorda e esteatose hepática, sugerindo assim que essa levedura pode também atuar como um tratamento probiótico benéfico no contexto de obesidade e diabetes tipo 2.

O consumo de probiótico é útil no tratamento de muitos tipos de diarreia, incluindo a diarreia associada a antibióticos, diarreia do viajante, e as doenças diarreicas em crianças pequenas causadas por rotavírus. Também está associado ao controle de doenças inflamatórias, doenças intestinais e síndrome do intestino irritável, proteção contra câncer de cólon e bexiga (KATHLEEN, 2010).

Leveduras têm sido investigadas quanto à sua capacidade para assimilar o colesterol e redução do seu nível, estudos demonstraram que a remoção de colesterol do de leveduras em caldo YEGP estava relacionada a absorção pelas células de levedura em crescimento (PSOMAS et al., 2003). *S. boulardii*, *P. kudriavzevii* e *S. cerevisiae* foram avaliados como potenciais probióticos para assimilar o colesterol ao longo de alguns anos (PSOMAS et al., 2001). Estudos mais recentes selecionaram cepas de *Pichia fermentans*, *P. kudriavzevii* e *Yarrowia lipolytica* como potenciais probióticos na sua redução (CHEN et al., 2010b). Cepas de bactérias láticas também demonstraram efeito na redução em pacientes com alto índice de colesterol (FUENTES et al., 2012).

É interessante também avaliar propriedades funcionais adicionais em leveduras testadas para características probióticas, tais como a atividade antioxidante (CHEN et al., 2010a). Evidências indicam que muitos processos patológicos como artrite reumatóide, doenças cardiovasculares e alguns tumores, são secundários a um dano oxidativo produzido pela presença de radicais livres, especialmente espécies reativas de oxigênio (ROS) (KAWAGISHI; FINKEL, 2014). Portanto, há grande interesse no estudo de antioxidantes naturais. Foi relatado recentemente que leveduras e extratos de levedura têm atividade antioxidante devido a vasta gama de substâncias presentes como, por exemplo, presença de superóxido dismutase, catalase, carotenóides oxigenados, entre outros. E também pela presença de (1/3) -β-D-glucano e outros β-glucanos encontrados em sua parede celular (GIL-RODRÍGUEZ; CARRASCOSA; REQUENA, 2015).

Os probióticos podem induzir efeitos fisiológicos não somente em pacientes doentes, mas também em pessoa considerada saudável. Estudos demonstraram que o consumo de *Lactobacillus paracasei* em adultos saudáveis resultava em aumento de espécies de *Lactobacillus*, *Bifidobacterium*, e *Roseburia intestinalis* e diminuição da bactéria *Escherichia coli* (ZHANG et al., 2013).

2.5 Leveduras como probióticos

As leveduras são microrganismos eucarióticos, encontradas em ambientes naturais, incluindo a microbiota residente dos seres humanos, em plantas, em partículas transportadas pelo ar, na água, nos alimentos, e em muitos outros nichos ecológicos (HATOUM; LABRIE; FLISS, 2012). Elas são importantes em muitos ecossistemas, como colonizadoras iniciais de substratos ricos em nutrientes.

Desde muito tempo, os seres humanos têm explorado leveduras e seus produtos metabólicos, principalmente para fabricação de cerveja e pão. Hoje em dia, muitos setores comercialmente importantes, incluindo alimentos, bebidas, produtos farmacêuticos, enzimas, industriais e outros dependem desses microrganismos. Elas também desempenham importante papel na fermentação espontânea de muitos produtos alimentares (MOSLEHI-JENABIAN; PEDERSEN; JESPERSEN, 2010).

Entre as leveduras pertencentes ao filo Ascomycota, o gênero *Saccharomyces* é o mais estudado, já que maioria possui interesse em aplicações biotecnológicas que inclui fermentação alcoólica, panificação, como proteínas de célula única, produção de vitamina, a síntese de proteínas recombinantes e no controle biológico (HATOUM; LABRIE; FLISS, 2012).

As leveduras chamaram a atenção para a sua possível utilização como probióticos, devido a sua capacidade de sobreviver durante a passagem pelo TGI humano, assim como por tolerar a exposições em baixo pH e a sais biliares (LOURENS-HATTINGH; VILJOEN, 2001).

Outro fator é o desenvolvimento da resistência antimicrobiana das bactérias patogénicas associadas com o tratamento por antibiótico, o que tornou um problema de saúde pública. Genes de resistência podem ser transferidos não só entre os membros da microbiota, mas também entre os probióticos e patógenos. Genes de resistência a antibióticos já foram relatados em várias cepas de *Lactobacillus.* A transferência de material genético não ocorre entre bactérias e leveduras, tornando leveduras seguras para uso durante o tratamento de pacientes com antibióticos (CZERUCKA; PICHE; RAMPAL, 2007).

Silva et al. (2011) relataram a presença de leveduras probióticas em azeitonas salgadas portuguesas, enquanto, Pedersen et al.(2012) estudaram as leveduras com características probióticas isoladas de Fura, um fermentado tradicional de cereais africano. Leveduras

probióticas também foram encontradas em um alimento fermentado da Itália e do Himalaia (SOURABH, KANWAR, SHARMA, 2011; PERRICONE et al., 2014).

A maioria dos probióticos estudados ou comercializados hoje foi selecionada a partir do ecossistema digestivo, alguns destes também foram isolados de outros ambientes não intestinais, como exemplo *Saccharomyces boulardii*, que é uma levedura não patogênicas isolada de frutos de lichia (MARTINS et al., 2005; MCFARLAND; BERNASCONIG, 1993). Ela possui diversas propriedades que a tornam potencial agente probiótico, ou seja, sobrevive à passagem do trato gastrointestinal, sua temperatura ótima é de 37º C. Tanto *in vitro* como *in vivo* ela inibe o crescimento de agentes patogénicos microbianos (CZERUCKA; PICHE; RAMPAL, 2007).

A sua eficácia foi demonstrada no tratamento de diarreia do viajante, diarreia que ocorre em indivíduos infectados como vírus da imunodeficiência humana, a prevenção de diarreia associada a antibióticos, e na profilaxia de *Clostridium difficile*. Estudos em modelos animais têm avaliado também os efeitos de *S. boulardii* em doença inflamatória do intestino, em modelo de colite induzida, a administração de *S. boulardii* reduzia as características clínicas da inflamação e diminuía os níveis de citocinas pró-inflamatórias nas mucosas (BOURREILLE et al., 2013; LEE et al., 2009).

O pH do estômago varia de 2,5 a 3,5 não é favorável a maioria dos microrganismos, porém, algumas espécies de leveduras são encontradas no estômago e cólon, sua presença pode ser explicada pela resistência à variação de pH. A maioria das leveduras podem crescer em pH 3,0, e algumas espécies podem tolerar condições altamente ácidas (CZERUCKA; PICHE; RAMPAL, 2007).

O sistema de força próton-motriz é a fonte central de energia e o gradiente de pH na membrana celular (KRULWICH; SACHS; PADAN 2011). Em levedura, a enzima H^+ ATPase presente na membrana plasmática, codificada pelo gene PMA1, gera um gradiente eletroquímico de próton que impulsiona o transporte secundário ativo e regula pH intracelular e extracelular. Esta bomba de prótons é ativada pela acidificação intracelular e sua atividade é crucial para que os microrganismos tolerem o estresse ácido (HUESO; GARCÍA; HERNÁNDEZ, 2012).

Leveduras também têm demonstrado uma alta capacidade de auto-agregação (se agregarem) esta capacidade é um requisito importante para que as células não sejam imediatamente eliminadas, devido a movimentos peristálticos (SYAL; VOHRA, 2013). Essa auto-agregação pode estar associada com algumas propriedades físico-químicas da superfície

celular de cada microrganismo, como por exemplo, a presença de glicoproteínas (lectinas) na superfície celular (KOS et al., 2003). Assim como a auto-agregação a co-agregação é um fator importante para seleção de novas cepas probióticas, leveduras do gênero *Saccharomyces*, conseguem se aderir aos microrganismos patogênicos na parede celular, com a formação de um “complexo levedura-bactéria”, que aumenta a susceptibilidade aos mecanismos de eliminação de patógenos (ROSTAGNO, 2003).

Além da levedura *S. boulardii*, várias outras espécies, incluindo *Debaryomyces hansenii*, *Torulaspora delbrueckii* (PSANI; KOTZEKIDOU, 2006), *Kluyveromyces marxianus*, *K. lodderae* (KUMURA et al., 2004) têm sido encontradas como fortes antagonistas a bactérias patogênicas e por sua capacidade de tolerar a passagem através do TGI. Porém a única levedura probiótica disponível comercialmente é a *S. boulardii*, já que sua eficiência probiótica foi documentada em vários estudos clínicos (CZERUCKA; PICHE; RAMPAL, 2007).

Estudos demonstraram que estirpes de *S. cerevisiae* são mais resistentes do que as não-*Saccharomyces*, e que a cepa comercial *S. boulardii*, não foi uma das melhores leveduras já avaliadas (ARÉVALO-VILLENA et al., 2018).

2.6 Alimentos fermentados, fonte de potenciais probióticos

Alimentos e bebidas fermentados são produtos do cotidiano e da dieta de muitas pessoas, foram produzidos e consumidos desde o desenvolvimento das civilizações. Alimentos Fermentados são geralmente definidos como alimentos ou bebidas que sofrem ação através de crescimento microbiano controlado e conversões enzimáticas destes (MARCO et al., 2017).

Grãos de cacau é a principal matéria-prima na fabricação de chocolate. Há diferentes etapas envolvidas no processamento do cacau. O processo de fermentação é a primeira etapa, nesse processo estão envolvidos microrganismos, como as leveduras, bactérias do ácido láctico e bactérias de ácido acético. (ARDHANA E FLEET, 2003; MOREIRA et al., 2013).

A polpa de cacau possui um baixo pH, entre 3,0-3,5, esse baixo pH é associado com o elevado teor de ácido cítrico encontrado na polpa. A condição ácida favorece o crescimento de leveduras nas primeiras 24 h de fermentação (SCHWAN, 1998).

Alguns estudos têm mostrado que bactérias presentes na fermentação de cacau (especialmente *Lactobacillus*) podem ser potencias probióticos, apresentando tolerância e

viabilidade às condições do TGI (FOONG et al., 2013; RAMLI et al., 2012; RAMOS et al., 2013; SAITO et al., 2014).

O kefir é obtido pela inoculação de leite com grãos de kefir. Estes grãos são irregulares, podem variar de tamanho de 3 a 35 mm de diâmetro e contêm bactérias láticas (*Lactobacillus*, *Lactococcus*, *Leuconostoc*), bactérias acéticas (*Acetobacter*) e leveduras (MAGALHÃES et al., 2010).

As leveduras são importantes na fermentação por desempenharem um papel fundamental na formação do sabor e aroma. Os grãos de kefir geralmente contêm leveduras fermentadoras de lactose, como *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Pichia fermentans*, *Yarrowia lypolytica* e *Torula kefir*, assim como leveduras não fermentadoras da lactose, como a *S. cerevisiae*. Análises da microbiota de amostras de kefir feita a partir de diferentes locais indicam populações diferentes entre as amostras (SIMOVA et al., 2002; FARNWORTH, 2005).

Estirpes expressando características únicas e particulares que possam proporcionar benefícios para a saúde podem surgir na caracterização de produtos lácteos fermentados naturais, como o kefir (LEITE et al., 2015).

Assim como cacau e kefir, as bebidas fermentadas produzidas por fermentação em pequena escala pelas tribos indígenas possuem um alto valor nutricional, além de ser importante nos rituais indígenas e comemorações. O cauim, tarubá, calugi, caxiri e yakupa são exemplos dessas bebidas indígenas (SANTOS et al., 2012; MIGUEL et al., 2014; FREIRE et al., 2014; RAMOS et al., 2015). Santos et al. (2012) estudando a comunidade microbiana presente no caxiri, indicou que a levedura era o grupo dominante e que *S. cerevisiae* foi a espécie de levedura predominantes, enquanto *Rhodotorula mucilaginosa*, *Pichia membranifaciens*, *P. guilliermondii* e *Candida luteolus* foram encontradas, mas com menor incidência. Além de leveduras foram encontradas espécies bacterianas como *Bacillus* e *Lactobacillus*, esses três grupos de microrganismos vivem em associação simbiótica.

Estudos demonstraram que cepas de *Lactobacillus brevis* isolados do cauim, são potenciais probióticos (RAMOS et al., 2013). Há também estudos que demonstraram a presença de leveduras com potenciais probióticos encontrados em fura, produto espontaneamente fermentado de milho, produzido na África (PEDERSEN et al., 2012b).

Em geral, o potencial probiótico de microrganismos associados aos produtos espontaneamente fermentados é mais investigado para o grupo de bactérias, principalmente

bactérias do ácido lático. No entanto, conforme descrito acima, leveduras também estão presentes nestes produtos e podem apresentar propriedades probióticas.

2.7 Produtos probióticos não lácteos

Os microrganismos probióticos são comercializados principalmente na forma de alimentos fermentados, na sua grande maioria, produtos lácteos, porém o desenvolvimento de novos produtos alimentícios na categoria de bebidas funcionais alternativas à base de plantas ou produtos não lácteos é um segmento crescente no mundo, isso devido a alguns fatores, como à alta intolerância à lactose, cerca de 75% da população mundial possui intolerância (SILANIKOVE; LEITNER; MERIN, 2015), alergia aos derivados do leite, como a caseína e também pelas dietas veganas (COSTA et al., 2017; SETHI; TYAGI; ANURAG, 2016).

Alternativa ao leite tem sido buscada, uma dessas, seria o extrato solúvel à base de plantas, que são fluidos resultantes da redução de tamanho do material vegetal, seja ele, cereais, leguminosas, oleaginosas, estes são extraídos em água.

Os cereais são importantes fontes de proteínas, carboidratos, vitaminas, minerais e fibras em todo mundo. Podem ser usados como fontes de carboidratos não digeríveis que além de promover vários efeitos fisiológicos benéficos também estimulam crescimento de microrganismos como lactobacilos e bifidobactérias presentes no cólon. O extrato solúvel à base de cereais pode ser: de aveia, arroz, milho; de leguminosas: de soja, amendoim; de nozes: amêndoa, coco, avelã, pistache, noz; e sementes, como o linho e girassol (SETHI; TYAGI; ANURAG, 2016).

Alguns estudos avaliaram a adição de culturas probióticas a bebida fermentada obtida a partir de arroz (HASSAN; ALY; EL-HADIDIE, 2012) e aveia (MÅRTENSSON et al., 2002). A sobrevivência de microrganismos probióticos também foi relatada em substrato fermentado à base de milho (HELLAND; WICKLUND; NARVHUS, 2004). A mistura de cereais com outros substratos, como por exemplo, mandioca e arroz (FREIRE et al., 2017), milho e arroz (FREIRE; RAMOS; SCHWAN, 2017), leite de amendoim com soja (SANTOS; LIBECK; SCHWAN, 2014), soja e arroz (COSTA et al., 2017) têm sido também utilizadas para produção de alimentos probióticos.

Além de produtos probióticos a base de cerais, outros estão ganhando interesse, como a crescente tendência por produtos de confeitaria que proporcionam benefícios funcionais a saúde e bem-estar, como os chocolates (KONAR et al., 2016), além de produtos à base de

água de coco (KANTACHOTE et al., 2017) e sucos de frutas. As frutas são saudáveis e possuem características refrescantes com boa aceitação (LUCKOW; DELAHUNTY, 2004; PANGHAL; VIRKAR; KUMAR, 2017). O setor de processamento de carne vem investindo no desenvolvimento e comercialização de produtos de carnes funcionais, como por exemplo, salsichas fermentadas com cepas probióticas (RUIZ-MOYANO et al., 2011).

2.8 Redução de fitato em produtos a base de vegetais

O fósforo é armazenado em cereais, legumes, sementes oleaginosas, forragens e raízes como fitato e fitina, durante o amadurecimento, cereais e sementes de leguminosas acumulam uma quantidade substancial de ácido fítico, o que representa de 60% a 80% do total de fósforo que só podem ser digeridos por ruminantes. Não ruminantes, como os porcos, aves e seres humanos são incapazes de utilizar, sendo assim, fósforo inorgânico é adicionado às dietas desses animais para suplementar os requisitos nutricionais de fósforo (LEI E PORRES, 2003; RIES E MACEDO, 2009).

Além disso, o ácido fítico age como um antinutriente, pois se liga a metais importantes em termos nutricionais, como ferro, zinco, magnésio e cálcio, assim como proteínas e lipídios, diminuindo a biodisponibilidade desses nutrientes (HOWSON; DAVIS, 1983).

A enzima fitase catalisa a hidrólise do fitato, aumentando a digestibilidade das proteínas e a disponibilidade de fósforo e outros minerais, que geralmente formam complexos com o ácido fítico (KONIETZNY; GREINER, 2002).

Além das plantas, os microrganismos, incluindo bactérias, leveduras e espécies de fungos também produzem fitase. Normalmente, as fitases produzidas por fungos são extracelulares, ao contrário das enzimas de bactérias (KONIETZNY; GREINER, 2002).

Leveduras foram relatadas como microrganismos produtores de enzima fitase, espécies de *Saccharomyces cerevisiae* e *Pichia kudriavzevii* são bem conhecidos por serem boas produtoras de fitase. A adição de fitases ou microrganismos produtores dessa enzima em alimentos poderia reduzir o teor de fitato, aumentando assim a disponibilidade de nutrientes aumentando seu valor nutricional (NUOBARIENE et al., 2012; RIES E MACEDO, 2009; FISCHER et al., 2014).

3 CONSIDERAÇÕES FINAIS

O aumento da consciência dos consumidores que buscam por melhorar a qualidade de vida e que optaram por hábitos saudáveis, vem aumentando consideravelmente. Neste contexto, o conceito de alimentos vem mudando, não são tidos apenas como fonte de energia, tem-se demonstrado que a nutrição desempenha um importante papel na prevenção de doenças e promoção da saúde. Devido a isso, o estudo de características probióticas de diferentes microrganismos têm se tornado de grande importância. Microrganismos probióticos são amplamente adicionados em produtos alimentícios no intuito de trazer benefícios à saúde. Alimentos fermentados espontaneamente são considerados fontes de microrganismos cujas atividades funcionais ainda são pouco conhecidas, principalmente de leveduras, que são potenciais candidatas a probióticos por sobreviverem às condições severas do TGI. Porém, estas também não devem interferir na qualidade do alimento a que forem adicionadas, bem como, devem ser manter viáveis em condições de armazenamento. Nesse contexto, a seleção de leveduras potencialmente probióticas provenientes de fermentações naturais de alimentos tradicionais brasileiros, assim como a utilização destas para produção de novos produtos, através da fermentação de substratos, como cereais, podem ser alternativas interessantes para obtenção de bebida fermentada probiótica não láctea.

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SEGUNDA PARTE- ARTIGOS

ARTIGO 1- In vitro characterization of indigenous yeasts isolated from Brazilian food fermentations for potential use as probiotics

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Abstract

While many bacteria have been used as probiotics by industries, only two yeasts, *Saccharomyces cerevisiae* var. *boulardii* and *Kluyveromyces fragilis* (B0399), have been used for this purpose. In the present work, a total of 116 yeasts isolated from Brazilian indigenous fermented food, cocoa fermentation and kefir were *in vitro* characterized for probiotic attributes. From 116 isolates, 36 were tolerant to gastrointestinal conditions evaluated by tolerance to pH 2.0, bile salts (0.3% w/v), and 37°C temperature. From those, 15 isolates showed a similar or higher percentage ($P<0.05$) of hydrophobicity, autoaggregation and coaggregation with *E. coli* than the reference strain *S. boulardii*. All these strains showed a high percentage of adhesion to Caco-2 cells (> 63%) and antioxidant activity (ranging from 18 to 62%). Phytate hydrolysis was evaluated for these yeasts and 13 strains showed positive results, which is important for nutrient availability in plant-based foods. These results are important insights for characterization of novel probiotic yeast strains as well as to aggregate functional value to these food products.

Keywords: Probiotics; phytase; antioxidant activity; adhesion; Caco-2.

1 Introduction

Nowadays, more and more attention is being paid to the development of functional foods that contain probiotic microorganisms for health promotion and disease prevention. Probiotics are described as live microorganisms that confer a beneficial effect on the host when administered in the proper amount [1–3].

In general, most of the probiotic strains described and commercialized belong to the lactic acid bacteria (LAB) group, especially *Bifidobacteria* sp. and *Lactobacillus* sp. *Saccharomyces cerevisiae* var. *boulardii* and *Kluyveromyces fragilis* (B0399) are the only probiotic yeast species commercially available for human use [4, 5]. Yeasts, mainly *Saccharomyces cerevisiae*, have been extensively studied and used in industries due to their safe status and technological applications, such as in the bakery and brewery industries. The beneficial effects of *Saccharomyces cerevisiae* and non-*Saccharomyces* strains (e.g. *Debaryomyces*, *Torulaspora*, *Kluyveromyces*, *Pichia* and *Candida* genera) on human health have been demonstrated and described by several authors [6–8].

Fermented foods (e.g. cocoa, sausage, cauim, kefir, and others) have been shown to be a reservoir of new potential starter cultures and probiotic microorganisms, especially LAB [9–11]. Some authors have performed characterization of probiotic yeasts from fermentations. Perricone et al. [12] performed a technological and probiotic characterization of yeasts isolated from Altamura sourdough. Silva et al. [8] studied probiotic properties of yeasts from Portuguese brined olives, and Pedersen et al. [13] evaluated the adhesion capacity of different cell lines of yeasts from fura, a traditional African fermented cereal. However, data about probiotic potential of yeasts from indigenous fermented foods produced in Brazil are still scarce. Although the literature about spontaneous fermentations in food production has increased in the last few decades, most of these occurrences are attributed to microbial composition, chemical characterization and some technological starter culture use have been

proposed as reasons for these results [11, 14–18]. As it is known that yeasts have been repeatedly isolated from different indigenous fermentations for food preparation and that they may have important functional properties, the aim of this work was to evaluate the probiotic and functional characteristics of yeast strains isolated from Brazilian spontaneous fermentations (cocoa, kefir and indigenous food fermentations).

2 Materials and methods

2.1 Yeast strains and growth conditions

A total of 116 (Suppl. Table 1) yeast strains belonging to the Culture Collection of Agricultural Microbiology (CCMA) of the Federal University of Lavras and isolated from different fermentation processes; cocoa (60 isolates), kefir (14 isolates) and indigenous beverages (42 isolates) were studied. Strains were cultivated in yeast extract glucose-peptone medium (YPG) (2% w/v peptone, 1% w/v yeast extract, 2% w/v glucose) broth at 30°C for 24 h for the assays and were maintained as frozen stocks at -80°C in the presence of 20% (v/v) glycerol as a cryoprotective agent.

The pathogenic strain *Escherichia coli* (EPEC) CDC 055, provided by the Microbiology Laboratory of the Department of Food Science in the Federal University of Lavras, was employed in the coaggregation assay (section *2.6*). For the assay, *E. coli* was grown in Brain Heart Infusion (BHI; Oxoid, Roskilde, Denmark) broth at 37°C for 24 h.

The yeast *Saccharomyces boulardii* (Floratil®, Merck, Darmstadt, Germany) was employed as a reference strain for all assays. This yeast was reactivated in yeast extract peptone dextrose (YPD) broth and subcultured at 30°C for 24 h for the assays, as performed for the other yeast strains.

2.2 Tolerance to pH 2.0 and temperature of 37°C

The 116 isolates were subjected to tests of tolerance to pH 2.0 and 37 °C according to Ramos et al. [9] in order to select the resistant isolates for further studies. Yeast cells cultivated in YPG broth at 30°C for 24h were centrifuged (5000 rpm for 5 min at 4°C) and resuspended in YPG broth (Merck, Darmstadt, Germany) with pH adjusted to 2.0 using 1N HCl and incubated for 3 h at 37°C. Samples (10 µL) were obtained at time 0 and after 3 h and plated on YPG agar. Tolerance to pH 2.0 and 37°C temperature was indicated by subsequent growth on YPG agar plates after 24 h of incubation at 37 °C.

2.3 Determination of bile tolerance

The bile salts tolerance was investigated according to the methodology used by Pedersen et al. [13]. The percentage of yeast growth was determined by the formula (Abs/Abs_c) x100 according to Bevilacqua, Perricone et al. [19], where “Abs” is the absorbance at 0.3% (w/v) bile salts, and “Abs_c” is the absorbance of the positive controls. The yeasts were classified as++++: >140% of the growth rate; +++: 100–139% of the growth rate; ++: 60–99% of the growth rate; +: 20–59% of the growth rate; ±: 1–19% of the growth rate; and –: 0% of the growth rate [13].

2.4 Hydrophobicity

The cell surface hydrophobicity was evaluated by measurement of microbial adhesion to solvents (MATS) as the ability of cells to bind to hydrocarbons, according to the methodology used by Binetti et al. [20]. The n-hexadecane was used as solvent and the cell surface hydrophobicity (H%) was calculated with the formula $\% \ H = [(OD_0\text{-}OD)/D_0] \times 100$, where OD_0 and OD are the optical density before and after extraction with n-hexadecane,

respectively. Many authors have reported that a hydrophobicity of 30–40% could suggest the ability of probiotics to interact with mucus and perform at least a transient adhesion [21, 22].

2.5Autoaggregation ability

The autoaggregation ability was performed according Kos et al. [23]. Autoaggregation ability percentage was calculated using the formula Au% = 1- (OD_f/OD_0) x 100, where 'OD_0'and 'OD_f' are the optical density before and after incubation, respectively. Autoaggregation ability above 80% is considered strong [24].

2.6 Coaggregation with pathogens

The coaggregation assay was performed according to Kos et al. [23] with some modifications. *Escherichia coli* CDC 055 was used as a pathogen. The method for preparing the cell suspensions for coaggregation was the same as for the autoaggregation assay. Equal volumes (2 mL) of each cell suspension were mixed together in pairs by vortexing for 10 s. Tubes containing 4 ml of each yeast or *E. coli* CDC 055 suspension were used as controls. The absorbencies (*A*) at 600 nm of the suspensions were measured after mixing, and after 3 hours of incubation at room temperature. Samples were taken in the same way as in the autoaggregation assay. The percentage of coaggregation was calculated using equation:

$$Coagregation\ (\%) = \frac{((Ax + Ay)/2) - A\ (x + y)}{(Ax + Ay)/2}\ x100$$

where x and y represent yeast and *E. coli* CDC 055, respectively, in the control tubes, and (x+y) the mixture.

2.7 Hemolytic activity

Hemolytic activity was determined by inoculating the yeast strains on blood agar plates (Laborclin, Paraná, Brazil) containing 5% defibrinated sheep blood after 48 h of incubation at 37°C. The development of a clear zone of hydrolysis around the colonies was considered as a positive result. Green-hued zones around the colonies (α-hemolysis) or did not produce any effect on the blood plates (γ-hemolysis) were considered non hemolytic. Strains displaying blood lyses zones around the colonies were classified as hemolytic (β-hemolysis) [26].

2.8 Antioxidant activity

To evaluate the antioxidant activity, the percentage of reduction of the 1,1 diphenyl-2-picrylhydrazyl (DPPH) radical was performed with the protocol described by Chen et al. [27] with some variations.

Briefly, 1 mL of yeast culture in YPG broth was harvested by centrifugation (12,000 rpm, 5 min), washed twice with a sterile solution of 0.9% NaCl; the resulting pellet was resuspended in 1 mL of the same solution. The cell suspension (800 µL) was transferred into a new tube, where 1 mL of a DPPH solution (0.2 mM in methanol) was added afterward. The mix was vortexed and then incubated for 30 min at room temperature in darkness. The reaction tubes were centrifuged (9000 rpm, 5 min) and 300 µL of the supernatant was transferred into 96-well plates to measure the absorbance at 517 nm. The percentage of DPPH reduction was calculated as follows equation:

$$\% = \left[\frac{1 - \mathrm{A(sample)}}{\mathrm{A(blanck)}}\right] x100$$

The antioxidant capacity was also assessed by Trolox Equivalent using a solution of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) in methanol (1 mM). A calibration curve ($y = -0.0096x + 1.0571$), in the range of 10-100µmol Trolox/mL was used for quantification and showed good linearity ($R^2 = 0.9987$).

2.9 Screening for potential hydrolyzing phytate

The hydrolyzing phytate was performed according to Ries & Macedo [28] with some modifications. The yeast strains were tested for phytase production in solid medium. The phytase screening medium agar was composed as follows: glucose 15.0 g/L; calcium phytate 1.0 g/L; $(NH_4)_2SO_4$ 5.0g/L; $MgSO_4{\cdot}7H_2O$0.5 g/L; KCl0.5 g/L; $FeSO_4{\cdot}7H_2O$ 0.01 g/L; $MnSO_4{\cdot}1H_2O$ 0.1 g/L; and agar 15.0 g/L. The pH was adjusted to 5.5 using 0.1 N NaOH. The inoculated plates were incubated at 30°C for 24 h. Phytase production was observed in the plates by the formation of a clear zone around the colony. The halo sizes were measured (mm) and were considered positive in those strains which showed halo diameter ≥15mm.

2.10 Adhesion of yeasts to Caco-2 cell lines

2.10.1 Growth and maintenance of mammalian cell lines

The Caco-2 cells provided by the Cell Bank of Rio de Janeiro (BCRJ, Rio de Janeiro) were grown in modified Eagle's minimal essential medium (MEM supplemented with 10% (v/v) heat inactivated fetal bovine serum), 1x non-essential amino acids, and 0.1 mg/mL gentamicin. All solutions were obtained from Invitrogen, Gibco (Naerum, Denmark). The cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. The culture media were changed routinely and once the cells reached sub-confluence (80-90%), they were subpassaged.

2.10.2 Adhesion assay on Caco-2 cells

For the adhesion test to the human colon adenocarcinoma cell line, Caco-2 was investigated for the 13 selected yeast isolates according to Ramos et al. [9] with minor modifications. The Caco-2 cells were subcultivated (2×10^5 cell/mL) in 24-well tissue culture plates (Sarstedt,

Germany) and grown at 37 °C in a humidified atmosphere of 5% CO_2 for 14 d to obtain differentiation [29] in cell media. The cell culture medium was changed on alternate days.

For the adhesion assay, yeasts were cultured in YPG 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 *section 2.9.1*) at a concentration of about 10^7 CFU/mL. One milliliter of each yeast suspension was added to the cell line culture in the well and incubated for 1h at 37 °C in a 5% CO_2 atmosphere. Afterward, 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 of incubation at 37 °C, the solution with released yeast cells was serially diluted and enumerated on YPG agar. The plates were incubated at 30°C for 24 h. Adhesion ability was expressed as the percentage ratio between the yeast counts initially seeded and the counts after the washing steps (log CFU/mL). Experiments were performed with triplicate determinations and repeated twice.

2. 11 Statistical analyses

Analyses of the variance and the Scott–Knott test were performed with SISVAR 5.1 software. A value of $P < 0.05$ was considered significant.

3 Results and discussion

3.1 Tolerance to low pH and bile salt at 37° C

Tolerance to low pH and bile salts, in addition to adaptation to corporal temperature (37°C), is considered a prerequisite for probiotic strains to exert their beneficial effects on the human gut [5]. On a pH lower than the pKa value of the organic acids, a major portion of the acid is in the non-ionized form, which is permeable through the cell membrane of microorganisms. Once non-ionized acid is inside the cell where the pH is near neutral, acid ionizes and hence,

cannot come out of the cells, in yeast, the H + ATPase enzyme present in the plasma membrane regulates intracellular and extracellular pH [30].

Thus, a total of 116 yeasts isolates obtained from spontaneous food fermentations (Suppl. Table 1) were exposed to acidic conditions and a temperature of 37°C, similar conditions to those found in the stomach. From those isolates, 83 strains were pH 2.0 tolerant at 37°C and were selected, including isolates belonging to *Kluveromyces marxianus (5)*, *Saccharomyces cerevisiae (44)*, *Pichia membranifaciens (1),Rhodotorula mucilaginosa (2)*, *Pichia guilliermondii (3),Candida metapsilosis (2), Pichia caribbica (1),Candida orthopsilosis (3),Candida azyma (1),Pichia kluyveri (10), Hanseniaspora uvarum (6), Candida lusitaniae (1), Hanseniaspora opuntiae (3),*and *Candida quercitrusa (1)*species. Resistance to acidic conditions was not surprising since the strains studied in this work were isolated from low pH environments such as kefir, cocoa fermentation and indigenous fermentation, in which they coexist with lactic and/or acetic acid-producing bacteria [6, 16, 17, 31, 32]. Among the selected strains, 36 were isolated from cocoa fermentation, 8from kefir and 39 from Brazilian indigenous beverages.

In addition to low pH tolerance, resistance to bile salts is also important, as they are lipid emulsifying agents that are released into the duodenum after ingestion of food, this bile salts, has antimicrobial activity. Any ingested microorganism will not reach the intestinal tract in a viable manner if it cannot withstand the presence of bile in the duodenum. Thus, the 83 selected isolates were exposed to bile salts at 0.3 % (w/v) (ox gall) for 3 h. All tested isolates were able to tolerate this condition; however, the growth and time of adaptation in the bile media was strain-dependent. Thus, from 83 isolates, 26 were well-adapted to bile salts medium (classified as ++++) and were selected for the next assays: *Kluveromyces marxianus (2)*, *Saccharomyces cerevisiae (15)*, *Pichia membranifaciens (1), Pichia guilliermondii (1),*

Candida orthopsilosis(2), Pichia kluyveri (1), Hanseniaspora uvarum (3) and *Candida quercitrusa (1).*

3.2 Hydrophobicity, autoaggregation andcoaggregation properties

In the present study, the 26 selected isolates were subjected to hydrophobicity, autoaggregation and coaggregation assays. The results are shown in Table 1. The ability of microorganisms to adhere to epithelial cells and mucosal surfaces is important in probiotic selections since it prolongs their permanence in the intestine and will allow them to exert healthy effects [33]. In general, the adhesion capacity of the microorganisms is a complex multistep process, which involves both electrostatic interactions and hydrophobic forces, specific interactions between the physical and chemical characteristics of the microbial surface and intestinal mucosa [30, 31]. Hydrophobicity and autoaggregation assays were employed as indirect and screening tools to test and select the potentiality of adhesion to intestinal mucosa by the isolates. This self-aggregation may be associated with some physical-chemical properties of the cell surface of each microorganism, such as the presence of glycoproteins (lectins) on the cell surface [23]. Furthermore, coaggregation of yeasts with *E. coli* CDC 055 was also evaluated. Coaggregation with potential pathogenic bacteria is an alternative mechanism for probiotic cultures to mechanically prevent a colonization of pathogens to the intestine.

All 26 isolates showed high hydrophobicity capacity (> 94%). Regarding the autoaggregation percentage, values ranged from 66.0 to 100.0 %. The lowest autoaggregation percentage ($P<0.05$) was shown for *Hanseniaspora opuntiae* CCMA 0579 (66.0%) while the highest ($P<0.05$) were shown for different yeast isolates (>88.0%) (Table 1). Both, auto-aggregation and hydrophobicity are strain-dependent based on cell wall composition [36].The coaggregation percentage of the yeast with *E. Coli* CDC 055 ranged from 0.9% to 76.2%,

showing significant differences (*P*<0.05) among the isolates. Comparing the yeast isolates to the reference strain *S. boulardii*, the highest percentages of hydrophobicity and autoaggregation found for yeast isolates did not differ statistically (*P*>0.05) for the reference strain. On the other hand, for the coaggregation assay, the reference strain was among the lowest (*P*<0.05) values found (6.31%). The yeasts studied displayed properties such as coaggregation with *E. coli,* higher than the commercial strain *S. boulardii*. Thus, a total of 15 isolates (*Saccharomyces cerevisiae* CCMA 0721, *Saccharomyces cerevisiae* CCMA 0491, *Saccharomyces cerevisiae* CCMA 0735, *Saccharomyces cerevisiae* CCMA 0731, *Pichia membranifaciens* CCMA 0016, *Saccharomyces cerevisiae* CCMA 0715, *Saccharomyces cerevisiae* CCMA 0712, *Saccharomyces cerevisiae* CCMA 0732¸ *Saccharomyces cerevisiae* CCMA 0716, *Saccharomyces cerevisiae* CCMA 0510, *Candida quercitrusa* CCMA 0560, *Pichia kluyveri* CCMA 0615, *Saccharomyces cerevisiae* CCMA 0739, *Saccharomyces cerevisiae* CCMA 0723, *Saccharomyces cerevisiae* CCMA 049) that showed similar or higher percentages (*P*<0.05) of hydrophobicity, autoaggregation and coaggregation with *E. coli* CDC 055 than the reference strain *S. boulardii* were selected for the following tests.

Table 1- Percentage (%) of hydrophobicity, autoaggregation andcoaggregation obtained for the different yeast isolates.

Yeast isolates	**Hydrophobicity (%)**	**Autoaggregation (%)**	**Coaggregation (%)**
Pichia guilliermondii CCMA 0019	94.9 ± 0.0^{a}	82.9 ± 2.3^{b}	ND
Kluveromyces marxianus CCMA 0686	98.9 ± 0.5^{d}	75.7 ± 5.4^{b}	ND
Candida orthopsilosis CCMA 0608	97.8 ± 0.0^{c}	88.1 ± 3.7^{c}	23.0 ± 2.9^{b}
Saccharomyces cerevisiae CCMA 0721	96.5 ± 0.0^{b}	96.7 ± 0.3^{c}	62.2 ± 3.3^{d}
Hanseniaspora uvarum CCMA 0603	97.5 ± 0.0^{c}	86.2 ± 0.4^{b}	7.1 ± 1.8^{a}

Saccharomyces cerevisiae CCMA 0491	99.6 ± 0.0^{e}	91.1 ± 4.2^{c}	56.8 ± 6.4^{c}
Kluveromyces marxianus CCMA 0688	100.0 ± 0.0^{e}	90.3 ± 4.1^{c}	4.3 ± 1.9^{a}
Hanseniaspora uvarum CCMA 0580	98.5 ± 0.9^{d}	83.7 ± 6.6^{b}	7.0 ± 1.6^{a}
Saccharomyces cerevisiae CCMA 0726	100.0 ± 0.0^{e}	98.7 ± 1.8^{c}	0.9 ± 0.7^{a}
Saccharomycescerevisiae CCMA 0735	100.0 ± 0.0^{e}	99.1 ± 0.7^{c}	48.7 ± 2.6^{c}
Saccharomycescerevisiae CCMA 0731	100.0 ± 0.0^{e}	99.1 ± 1.6^{c}	55.7 ± 0.7^{c}
Pichia membranifaciens CCMA 0016	99.7 ± 0.1^{e}	93.1 ± 3.5^{c}	13.2 ± 2.9^{b}
Saccharomyces cerevisiae CCMA 0508	100.0 ± 0.0^{e}	88.6 ± 0.3^{c}	2.8 ± 1.8^{a}
Candida orthopsilosis CCMA 0565	100.0 ± 0.0^{e}	87.1 ± 0.6^{b}	1.8 ± 0.8^{a}
Saccharomyces cerevisiae CCMA 0715	100.0 ± 0.0^{e}	92.9 ± 1.0^{c}	47.3 ± 0.4^{c}
Saccharomyces cerevisiae CCMA 0712	99.5 ± 0.1^{e}	95.0 ± 4.3^{c}	12.6 ± 0.8^{b}
Saccharomyces cerevisiae CCMA 0732	100.0 ± 0.0^{e}	95.9 ± 3.5^{c}	76.2 ± 5.3^{d}
Saccharomyces cerevisiae CCMA 0716	99.1 ± 0.0^{d}	99.3 ± 0.6^{c}	33.7 ± 5.2^{b}
Saccharomycescerevisiae CCMA 0510	100.0 ± 0.0^{e}	96.2 ± 1.0^{c}	10.9 ± 1.1^{b}
Candida quercitrusa CCMA 0560	98.9 ± 0.0^{d}	95.5 ± 3.6^{c}	22.6 ± 3.5^{b}
Saccharomyces cerevisiae CCMA 0727	99.9 ± 0.2^{e}	68.2 ± 1.8^{b}	32.9 ± 5.4^{c}
Pichia kluyveri CCMA 0615	99.3 ± 0.9^{d}	91.8 ± 0.4^{c}	40.9 ± 1.1^{c}
Saccharomyces cerevisiae CCMA 0739	98.9 ± 0.5^{d}	93.5 ± 2.3^{c}	69.1 ± 9.9^{d}
Saccharomyces cerevisiae CCMA 0723	100.0 ± 0.0^{e}	98.7 ± 0.0^{c}	73.8 ± 2.0^{d}
Saccharomyces cerevisiae CCMA 0493	99.3 ± 0.9^{d}	98.5 ± 1.3^{c}	21.3 ± 3.0^{b}
Hanseniaspora opuntiae CCMA 0579	98.7 ± 0.1^{d}	66.0 ± 2.1^{a}	3.7 ± 0.4^{a}
Saccharomyces boulardii	99.4 ± 0.2^{d}	95.5 ± 0.1^{c}	6.3 ± 1.9^{a}

ND = not determined by showing very low percentage. Presented values are means of duplicate determinations; ± indicates standard deviations from the mean. Mean values (±

standard deviation) within the same column followed by different superscript letters differ significantly ($P < 0.05$) by Scott–Knott test.

3.3 Hemolytic activity

Screening process for probiotic strains should consider safety aspects, including specifications such as origin, identity and lack of harmful activities [2]. In this study, no yeast strain was positive for hemolytic reaction, indicating that the isolates are safe to use.

3.4 Antioxidant activity

In addition to properties studied for probiotic traits, it is interesting to evaluate functional characteristics of yeasts, such as antioxidant capacity. Several studies have reported the natural antioxidant capacity of yeast cells [27, 37, 38]. Chen et al. [27] analyzed antioxidant activity for different yeast species, using intact cells and their extract. Their results demonstrate that intact cells had higher antioxidant capacity than the extract, which may be due mainly to the high content of (1 / 3) -β-D-glucan and other β-glucans found in their cell wall among other cellular compounds. In the present work, antioxidant activity of intact cells of the 15 selected yeast isolates (described above) were evaluated by the DPPH reduction method and all of them showed antioxidant capacity (Fig. 1). Gil-Rodríguez et al. [37] classified yeasts into 5 groups according to their percentage of antioxidant activity: very low (< 20%), low (20-30%), good (30-40%), very good (40-50%), and excellent (> que 50%). Based on this grouping, the yeasts studied in the present work were classified in all different levels: one isolate showed very low activity, four showed low activity, one showed good activity, four showed very good activity including the reference strain *S. boulardii*, and five showed excellent activity. Among all 15 isolates studied, *Saccharomyces cerevisiae* CCMA 0731 exhibited the highest percentage of DPPH reduction (61.6 ± 1.8), and *Saccharomyces*

cerevisiae CCMA 0735 exhibited the lowest value (18.3 ± 4.7). The antioxidant activity was also measured as Trolox Equivalent, obtaining activity intervals between 13.3 ± 1.9 (for *S. cerevisiae* 0735) and 61.05 ± 2.6 (for *S. cerevisiae* 0712). As previously observed by other authors [9, 26], microorganisms belonging to the same species may have different characteristics and show different properties, as observed for variations in percentage of antioxidant activity among the strains.

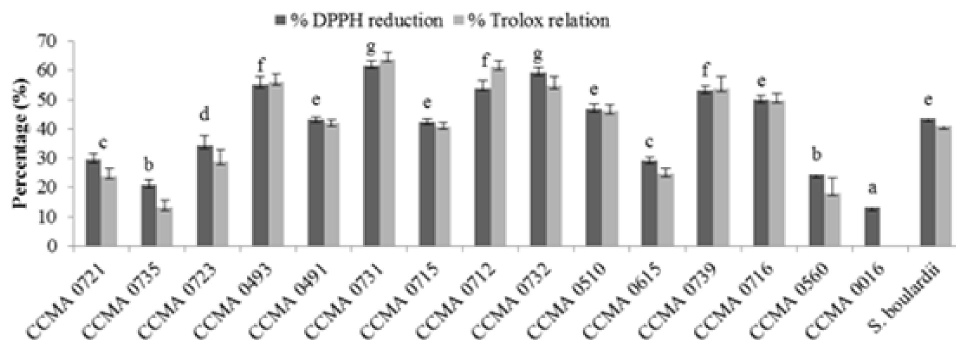

Figure 1. Antioxidant activity (%) of yeasts evaluated by DPPH reduction and Trolox equivalents.

3.5 Screening for potential hydrolyzing phytate

Phytate is an anti-nutrient present in cereals. It considerably reduces the bioavailability of other minerals, forming insoluble complexes with numerous cations such as copper, zinc, calcium, magnesium, iron and potassium. Furthermore, phytate may have a negative influence on the functional and nutritional properties of proteins [3]. Phytate complexes are not available for absorption in the human gut unless they are digested by enzymatic phosphatases such as phytase, which catalyze the phosphate hydrolysis from phytate.

The phytase enzyme is naturally synthesized in plants and some microorganisms. Yeasts have been reported as microorganisms useful for phytase production [39].The addition of phytase or microorganisms producing this enzyme in foods could reduce the phytate content, thus increasing the nutrient's availability [3, 40].

The 15 selected yeasts described above and the reference strain *S. boulardii* were evaluated for the ability to hydrolyze phytate by halo formation in solid media containing calcium phytate. All strains grew in the medium; however, only 14 (87.5%) were positive for the test as shown in Table 2. Ries & Macedo [28], studying yeasts isolated from soil, found that 70% of the strains showed phytase production at 30 °C evaluated by halo production in solid medium. Species of *Saccharomyces cerevisiae* and *Pichia kudriavzevii* are well known to be good phytase producers [41]. Nuobariene, Hansen, & Arneborg [41] found prominent production of extracellular phytase for *S. cerevisiae*, *Candida humilis* and *Pichia kudriavzevii* isolated from sourdoughs. According to our results (Table 2), all isolates of *S. cerevisiae* and *P. kluyveri*as well as the reference strain *S. boulardii* were phytase producers. *Candida quercitrusa* and *P. membranifasciens* were negative for the test.

Table 2- Phytate hydrolysis by yeasts strains evaluated by halo formation.

Strains	**Measure of halo (mm)**	**Hydrolyzing phytate**
Saccharomyces cerevisiae CCMA 0721	22.5 ± 3.5	+
Saccharomyces cerevisiae CCMA 0735	25.5 ± 3.5	+
Saccharomyces cerevisiae CCMA 0723	25.0 ± 1.4	+
Saccharomyces cerevisiae CCMA 0493	23.0 ± 1.4	+
Saccharomyces cerevisiae CCMA 0491	25.0 ± 0.0	+
Saccharomyces cerevisiae CCMA 0731	18.5 ± 0.7	+
Saccharomyces cerevisiae CCMA 0715	16.5 ± 3.5	+
Saccharomyces cerevisiae CCMA 0712	25.0 ± 4.2	+
Saccharomyces cerevisiae CCMA 0732	20.5 ± 6.4	+
Saccharomyces cerevisiae CCMA 0510	26.5 ± 2.1	+
Pichia kluyveri CCMA 0615	20.0 ± 1.4	+

Saccharomyces cerevisiae CCMA 0739	19.0 ± 0.0	+
Saccharomyces cerevisiae CCMA 0716	20.5 ± 2.1	+
Candida quercitrusa CCMA 0560	< 15.0	-
Pichia membranifaciens CCMA 0016	<15.0	-
Saccharomyces boulardii	20.0 ± 0.0	+

3.6 Adhesion ability to Caco-2 cell line

The ability to colonize the intestine, at least temporarily, through adhesion to the intestinal epithelium is an important criterion for selecting probiotic strains. Tumor cell lines of the human colon, such as Caco-2, are recognized as a good model for elucidating mechanisms involved in host microorganism interactions such as microbial adhesion to mucosa [42, 43]. The ability to adhere to Caco-2 cells was evaluated for the 13 selected yeasts described above for phytate hydrolysis, and the results are presented in Fig. 2. All yeast strains showed a high percentage of adhesion ability (ranging from70.7 to 89.3%). Comparing indigenous yeasts with the reference strain *Saccharomyces boulardii*, all strains showed similar (*P*>0.05) percentages of adhesion, except for the one isolate *Pichia kluyveri* CCMA 0615, which was lower (*P*<0.05) than the reference strain. The thirteen positive isolates followed the other tests.

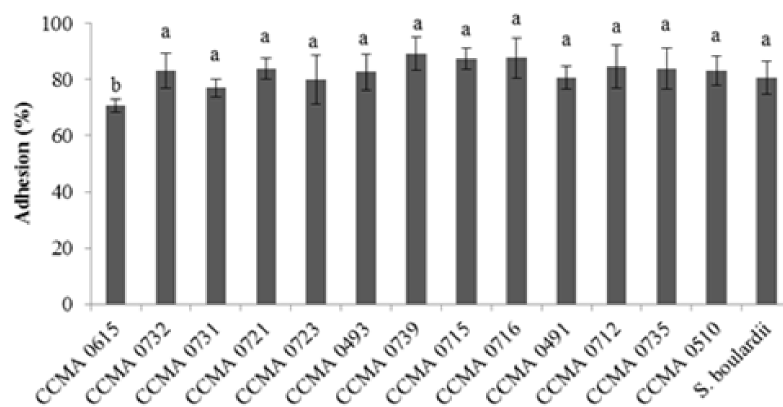

Figure 2. Percentage adhesion of yeasts to Caco-2 cells as calculated by plate count method.

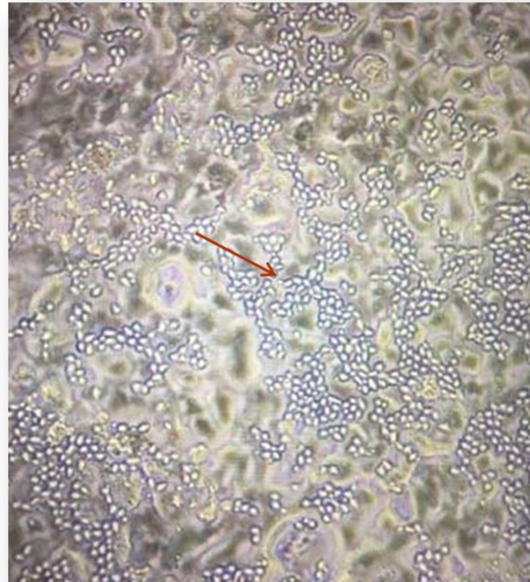

Figure 3. Adherence of yeasts to cells Caco-2 by optical microscopy.

5 Conclusion

Potential probiotic yeast isolates were characterized from different fermentations for foods production. Probiotic characteristics of yeast isolates from the Brazilian indigenous fermented foods and cocoa fermentation have not been studied before. It is an important find for characterization of novel probiotic strains as well as to aggregate functional value to these foods. From 116 isolates initially studied, twelve *S. cerevisiae* (9 from indigenous beverage caxiri and 3 from kefir) and one *P. kluyveri* (from cocoa fermentation) isolates showed potential properties to be used as probiotics. They tolerate low pH, bile salts, and 37°C temperature, conditions imposed by the gastrointestinal tract. Further, they showed high percentages of hydrophobicity, autoaggregation, coaggregation with *E. coli* and adhesion to Caco-2 cells, and their cells showed high antioxidant activity. In addition, these yeasts were able to produce phytase, an important enzyme for nutrient availability in plant-based foods. Among these selected isolates, four *S. cerevisiae* strains (CCMA 0739, CCMA 0712, CCMA

0731 from caxiri, and CCMA 0493 from kefir) displayed properties such as coaggregation with *E. coli* and antioxidant activity higher than the commercial strain *S. cerevisiae* var. *boulardii.* Although LAB is the preferred probiotic microorganism used by industries, yeasts may show other advantages such as high antioxidant activity. It was a first insight demonstrating the importance of searching for novel yeast strains with functional properties. Studies on the effect of the probiotic yeasts' addition to the fermentation for food production are the next step in developing novel, functional products.

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Conflicts of interest: none

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ARTIGI 2- Potential probiotic yeasts isolated from Brazilian fermented foods in the protection against infection by pathogenic food bacteria

Artigo redigido conforme norma do periódico International Journal of Food Microbiology, sujeito a modificações conforme sugestões do conselho editorial da revista.

Abstract

In recent years, considerable attention has been given to the use of probiotics. The consumption of probiotics has increased due to the reported health benefits, mainly in preventing or treating gastrointestinal pathology. The advantage of probiotics, as opposed to antibiotics, is that they do not select resistant bacteria or disturb the intestinal microbiota. By performing competition and exclusion assays, this study aimed to investigate the antimicrobial capacity of yeasts isolated from fermented foods (an indigenous beverage, kefir and cocoa) against the adhesion of foodborne pathogens to human intestinal Caco-2 cells. The adhesion tests showed that all the strains were able to co-aggregate with the tested pathogens; however, this activity was strain-dependent. These data were evident through quantitative analysis and with scanning electron and laser confocal microscopies. The inhibition tests showed that the adhesion of *Escherichia coli* EPEC, *Listeria monocytogenes* and *Salmonella* Enteritidis to Caco-2 cells was reduced by all the yeasts studied, and the lowest rate was obtained for EPEC. Most of the evaluated yeasts showed inhibition rates equal to or greater than the commercial probiotic yeast *Saccharomyces boulardii*. The yeasts were able to reduce up to 50% of the bacterial infection, for example *Pichia kluyveri* CCMA 0615 for EPEC in exclusion assay, *S. cerevisiae* CCMA 0731, 0732 and *P. kluyveri* 0615 for *L. monocytogenes* in exclusion and competition assay, *S. cerevisiae* CCMA 0731 in exclusion and *S. cerevisiae* 0731, 0732 and *P. kluyveri* 0615 in competition assay. The antimicrobial test confirmed that the mechanism to inhibit bacterial infection was the competition for nutrients and/or receptors in the intestinal mucosa. The studied yeasts showed promise as a protective or treatment culture in the attenuation of foodborne infections.

Keywords: Caco-2 cells, Foodborne pathogens, Competitive inhibition, Exclusion inhibition, laser confocal microscopies.

1 Introduction

The mucosal surface of the gastrointestinal tract (GIT) is susceptible to the adhesion and colonization of foodborne pathogens such as *Salmonella enterica*, *Listeria monocytogenes* and *Escherichia coli*, which can cause several diseases and even death (Saad, 2006). The administration of antibiotics is the most commonly adopted strategy to combat infections caused by these foodborne pathogens. However, the use of antibiotics can cause adverse effects, such as diarrhea and modification in the protective intestinal microbiota, which can result in a possible GIT colonization by pathogenic strains. In addition, the misuse and overuse of antibiotic agents have accelerated the antimicrobial resistance of pathogens. The administration of probiotics is already employed as an alternative clinical practice to combat pathogenic microorganisms (Crooks et al., 2012; Dubreuil et al., 2017).

Probiotics are living organisms that, when administered in adequate amounts, confer health benefits to the host (Fao et al., 2002). Previous research employing in vitro studies of intestinal cell models has shown that probiotics reduce the adhesion of pathogens and, consequently, their infection (Ait Seddik et al., 2017; Feng et al., 2015; Garriga et al., 2015; Jessie Lau and Chye, 2018). The majority of these studies are related to bacterial strains, especially the *Lactobacillus* genus. The use of yeast as a probiotic is still rarely studied, and *Saccharomyces boulardii* is the only commercialized strain, and the only one used in studies on pathogens (Czerucka et al., 2000).

There is growing interest in exploring the potential of probiotic strains that are active and stable and that confer health benefits due to their competitive advantage in colonizing the intestine, thereby exerting positive activity against enteropathogenic infections (Rokana et al., 2017). Several mechanisms of action have been proposed to explain how probiotics protect against bacterial infections, for example, by degrading toxins and receptors in the intestinal

mucosa, as well as the production of antagonistic compounds that diffuse in the medium and inhibit the growth of pathogens. However, these mechanisms are mainly described for bacteria, especially lactic acid bacteria; yeasts rarely showed these antagonistic abilities (Binetti et al., 2013; Silva et al., 2011). The main mechanism of probiotic yeast action is the adhesion to intestinal mucosa, which prevents the attachment of pathogenic microorganisms and, consequently, the invasion of intestinal epithelial cells (Binetti et al., 2013; Faghfoori et al., 2015; Tuo et al., 2018).

Although various in vitro intestinal cell models, including Caco-2 cells, have been used successfully to demonstrate the effect of probiotics on the adhesion of pathogenic microorganisms to these cells, very few have employed probiotic yeasts (Candela et al., 2008; Champagne et al., 2015; Feng et al., 2015; Woo and Ahn, 2013). The aim of this work was to evaluate potential probiotic yeasts strains, isolated from Brazilian food fermentation processes (the indigenous beverage Caxiri, kefir and cocoa fermentation), to combat foodborne infection based on the probiotic's interference with pathogen adhesion to Caco-2 cells. For this, quantitative analysis as well as scanning electron and laser confocal microscopies were employed.

2 Material and methods

2.1 Microorganisms

A total of 13 yeast strains (12 *Saccharomyces cerevisiae* isolated from kefir and the indigenous beverage Caxiri and one *Pichia kluyveri* isolated from cocoa fermentation) belonging to the Culture Collection of Agricultural Microbiology (CCMA) were previously characterized as potential probiotic yeasts (data not shown) and employed in this study. The commercial probiotic yeast *S. boulardii* (Floratil®) was used as a positive control.

The strains, including the commercial one, were grown in yeast extract glucose-peptone medium (YEPG) (Merck, Darmstadt, Germany) at 28°C for 24 h under constant stirring (120 rpm) for the assays and kept frozen at -80°C in the same medium with 20% (v/v) glycerol for stock cultures.

Escherichia coli EPEC CDC 055, *Listeria monocytogenes* ATCC 19117 provided by the Laboratory of Food Microbiology of the Food Science Department at the Federal University of Lavras (Lavras, MG. Brazil), and *Salmonella* Enteritidis S64, provided by the Osvaldo Cruz Foundation (FIOCRUZ, Rio de Janeiro), were grown in a brain-heart infusion (BHI) (Kasvi, Paraná, Brazil) at 37°C until a population of approximately 10^8 CFU/mL was reached for the assays. Stock cultures were kept frozen at -80°C in the same medium with 20% (v/v) glycerol.

2.2 Co-aggregation evaluation

To evaluate the yeast's ability to agglutinate bacterial pathogens, the method described by Tiago et al. (2012) was used with some modifications. One milliliter of each yeast culture previously grown in YEPG medium at 28°C for 24h (around 10^7 CFU/mL) was mixed with 500 mL of bacterial pathogens (EPEC, *L. monocytogenes* ATCC 19117 and *S.* Enteritidis ATCC 564) suspension at 10^8 CFU/mL, previously grown in BHI broth at 37°C, and inoculated in a 24-well plate. The presence or absence of agglutination was observed macroscopically by sedimentation after 3 h and by optical microscopy (Eclipse E200, Nikon, Japan) with gram-staining and were classified as follows: -: co-aggregation; +: low co-aggregation; ++: medium co-aggregation; +++: high co-aggregation.

2.3 Scanning electron microscopy (SEM) of yeasts co-aggregated with bacterial pathogens

Samples of positive co-aggregation of yeast and bacteria were collected, preserved in Karnovsky fixative solution (2.5% glutaraldehyde, 2.0% paraformaldehyde in 0.05 M sodium cacodylate buffer, 0.001 M $CaCl_2$, pH 7.2) and stored at 4°C for 24 h. After fixation, the samples were washed in 0.05 M cacodylate buffer three times for 10 minutes each, and transferred to a solution of 1% osmium tetroxide where they remained for 1 h. Afterwards, the samples were washed three times in distilled water and dehydrated in acetone gradient (25%, 50%, 75%, 90% and 100%) remained for 10 minutes in each concentration and three times for 10 minutes in the 100% solution, as described by Bozzola and Russel (1999). The samples were then dried in a Balzers CPD 030 (Balzers, Liechtenstein) critical point apparatus, where acetone was replaced by CO_2. The samples were covered with gold in sputtering Balzers SCD 050 (Balzers, Liechtenstein) for observation in a Zeiss LEO EVO 40 scanning electron microscope (SEM) (Carl Zeiss, Oberkochen, Germany). The generated images were edited using Corel Draw software.

2.4 Adhesion to Caco-2 cells

2.4.1 Growth and maintenance of Caco-2 cells

The Caco-2 cells, which were acquired from the Cell Bank of Rio de Janeiro (BCRJ, Rio de Janeiro, Brazil), were cultured in Modified Eagle Medium (MEM) supplemented 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, Gibco (Naerum, Denmark). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. The culture media were routinely exchanged for new media until the cells reached confluent monolayer formation (80%–90%) and then they were subpassaged for the assays plates.

2.4.2 Inhibition of pathogenic bacteria adhesion to Caco-2 by yeasts

The inhibition of the adhesion of the pathogens *L. monocytogenes*, EPEC and *S.* Enteritidis to Caco-2 cells by potential probiotic yeasts was performed according to Gueimonde et al. (2006) with minor modifications.

Yeast (10^7 CFU/mL) and pathogen (10^8 CFU/mL) were resuspended in 1 mL MEM (without fetal bovine serum and antibiotics) and were then added simultaneously to the Caco-2 cells after 14 days of culture (differentiated cells) in 24-well plates according to the following treatments: 1) inoculation of bacteria (10^8 CFU/mL) for 90 minutes (control); 2) inoculation of yeasts (10^7 CFU/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 CFU/mL) and bacteria (10^8 CFU/mL) for 90 minutes (competition). After the incubation, the cells were washed three times with phosphate-buffered saline (PBS) to remove non-adhered cells and then recovered by treatment with 1% (v/v) Triton-X for 10 min at 37°C. Bacteria were enumerated by plating in BHI medium. 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 = $((Np\text{-}Nmix) / Np) \times$ x100. The experiment was carried out in triplicate.

2.5 Inhibition of pathogens adhesion to Caco-2 cells evaluated by laser confocal microscopies

2.5.1 Fluorescent labeling of pathogenic bacteria with *Syto9*

The pathogenic bacteria were cultured, centrifuged and resuspended in 10 μM *Syto9* (Green-Fluorescent Nucleic Acid Stains, Molecular Probes), prepared in filtered dimethyl sulfoxide

(DMSO) and incubated for 30 min in the dark (Stiefel et al., 2015). After incubation, the cells were washed twice with 0.1 M potassium phosphate buffer pH 7.0 (PBS) followed by centrifugation at 4000 rpm for 10 min to remove excess of dye.

2.5.2 Laser confocal microscopies

Caco-2 cells cultured for 14 days in 24-well plates (differentiated cells) were incubated according to the treatments described above using the labeled cells (*Syto9* dye). Following the incubation period, the wells were washed twice with PBS and then taken for analysis using the Laser Confocal Microscope LSM780 Zeiss Observer Z.1 (Carl Zeiss Microscopy GmbH, Jena, Germany) using Zen software 2010. The detector ChS1, beam splitter MBS 488 nm, pinhole 60 µm, 20% laser, Master Gain 600, Digital Offset 0.0, Digital Gain 1.0, and Emission Filter for 518 at 544 nm were used. Images of the non-fluorescent cells were generated using the TPMT ChD detector, with Master Gain 210, Digital Offset - 970 and Digital Gain 1.0. The resolution was adjusted to 1024 × 1024 dpi, with 12 bits per pixel. EC Plan-Neofluar 10× / 0.30 Oil M27 objective was used with cropping images adjusted for 1 or 6× zoom. Approximately 5 focal capture planes (z-stack) were sampled at intervals of 3 µm between slices in a range of 12 µm. Subsequently, these focal planes were overlapped with the maximum intensity projection tool, resulting in a 2D final image with depth of field. Afterwards, the fluorescence intensity signal in the images was quantified using the mean intensity tool of the histogram screen in ImageJ software (Kask et al., 1999; Schneider et al., 2012).

2.6 Antimicrobial activity

Antimicrobial activity was evaluated by diffusion technique in the wells according to Psani and Kotzekidou (2006). The bacteria were grown at 37°C for 24 h in BHI, (Kasvi, Paraná

Brazil). The yeasts cells, cultivated in YEPG broth (Merck, Darmstadt, Germany) at 28°C for 24 h (10^7 cell/mL), were centrifuged (5000 rpm for 5 min at 4°C) and the supernatant was used for the test, 5 µL was added to the wells done on BHI and TSB media containing 100 µl of the pathogen (10^8 cell/mL). The plates were incubated at 37°C for 24 h.

2.7 Statistical analysis

The data were subject to analysis of variance (ANOVA), and the Tukey tests were performed with Prisma Graphpad software. A value of $p < 0.05$ was considered significant.

3 Results and discussion

3.1 Co-aggregation of yeasts and pathogenic bacteria evaluated by optical and scanning electron microscopy (SEM)

Table 1 shows the co-aggregation capacity observed for the different yeast strains. Among the evaluated yeasts, the strains CCMA 0731, CCMA 0732 and CCMA 0615 showed high co-aggregations with all tested pathogenic bacteria. To confirm and better visualize this interaction, SEM analyses were performed and are shown in Figure 1. High agglutination of pathogenic bacteria with the yeasts cells of CCMA 0731 and CCMA 0732 isolates from the indigenous fermented beverage and CCMA 0615 from cocoa fermentation, was confirmed by microscopy analysis and these strains were selected for the adhesion to Caco-2 cells assays.

Table1. Co-aggregation of pathogenic bacteria *Escherichia coli* EPEC, *Salmonella* Enteritidis and *Listeria monocytogenes* with different yeast strains.

Co-aggregation			
Yeast strains	EPEC	*S.* Enteritidis	*L. monocytogenes*
S. cerevisiae CCMA 0731	++	++	++
S. cerevisiae CCMA 0732	+++	++	++
P. klyveri CCMA 0615	+++	+	+++
S. cerevisiae CCMA 0723	++	+	+
S. cerevisiae CCMA 0735	-	+	+
S. cerevisiae CCMA 0712	+	-	+

S. cerevisiae CCMA 0739	++	-	-
S. cerevisiae CCMA 0715	++	-	-
S. cerevisiae CCMA 0510	+	-	-
S. cerevisiae CCMA 0493	+	-	+
S. cerevisiae CCMA 0721	+	+	-
S. cerevisiae CCMA 0491	+	+	-
S. cerevisiae CCMA 0716	-	-	-
S. boulardii	+	+	+

-: no co-aggregation; +: low co-aggregation; ++ medium co-aggregation; +++: high co-aggregation.

The ability of a yeast to bind to a pathogen varied according to the yeast species and the pathogenic bacteria. The yeast cell was agglutinated with several bacteria cells in a phenomenon called co-aggregation. In addition, self-aggregation of yeasts cells was also observed in the present study (Figure 1), which is a desirable characteristic for probiotic strains. The capacity to form cell aggregates increases the microbial adhesion to the intestinal mucosa, conferring advantages in the colonization of the GIT and reducing infections by pathogenic bacteria (García-Cayuela et al., 2014).

Self-aggregation is an important requirement for cells not to be immediately eliminated due to peristaltic movements (Syal and Vohra, 2013). This self-aggregation may be associated with some physical-chemical properties of the cell surface of each microorganism, such as the presence of glycoproteins (lectins) on the cell surface (Kos et al., 2003). As well as self-aggregation, co-aggregation is an important factor for the selection of new probiotic strains. Yeasts of the genus *Saccharomyces* adhere to the pathogenic microorganisms in the cell wall with the formation of a “yeast-bacteria complex” that increases susceptibility to the elimination mechanisms of pathogens (Rostagno, 2003).

As shown in Figure 1, there was lower co-aggregation of the yeast strains with the pathogenic specie *S.* Enteritidis. Similar results were obtained by Tiago et al. (2012) while evaluating *S. boulardii* 17 and *S. cerevisiae* UFMG 905, BY4741 and W303 strains.

Adhesion to the host epithelium is the initial and essential step for the establishment of bacterial infection (Xu et al., 2009). Thus, mechanisms that inhibit pathogen adhesion are of great interest in the prevention of several diseases. Probiotic microorganisms may exhibit important properties related to the inhibition of pathogen adhesion to the host cells, including competition for receptors in the target cell, production of a toxic environment, production of molecules with antimicrobial activity and co-aggregation with pathogens (Oelschlaeger, 2010). In this way, it would be possible to prevent and treat intestinal infections by ingesting probiotics.

Previous studies have shown that type I fimbriae are frequently involved in the adhesion of enteropathogenic bacteria, such as EPEC and *Salmonella*, to the epithelial surface, generally using mannose as a receptor. The yeast cell walls are rich in mannose, so probiotic yeasts may exert a protective effect by binding the pathogenic bacteria to their surface cells (Kline et al., 2009).

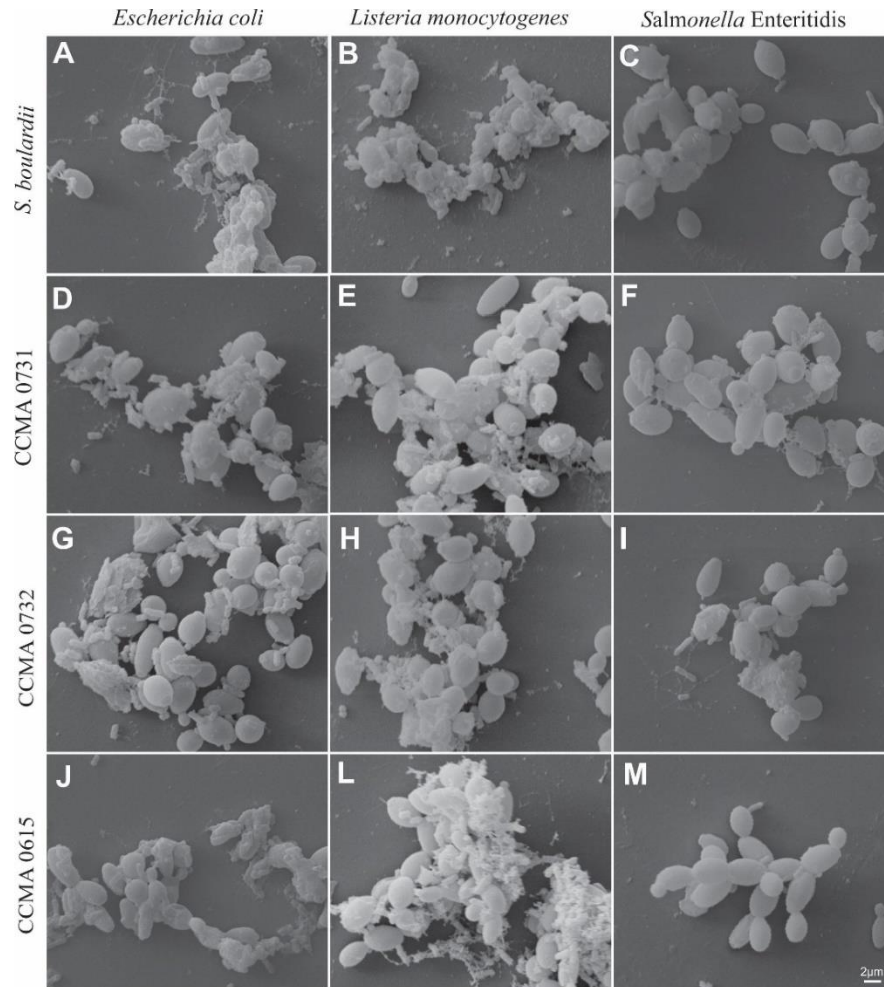


Fig. 1. Scanning electron micrographs of yeast cells co-aggregation and pathogenic bacteria strains evaluated by in vitro assay. Magnification is indicated on each panel. *Saccharomyces boulardii* and EPEC (A), *Saccharomyces boulardii* and *Listeria monocytogenes* (B), *Saccharomyces boulardii* and *Salmonella* Enteritidis (C), CCMA 0731 and EPEC (D), CCMA 0731 and *Listeria monocytogenes* (E), CCMA 0731 and *Salmonella* Enteritidis (F), CCMA 0732 and EPEC (G), CCMA 0732 and *Listeria monocytogenes* (H), CCMA 0732 and *Salmonella* Enteritidis (I), CCMA 0615 and EPEC (J), CCMA 0615 and *Listeria monocytogenes* (L), CCMA 0615 and *Salmonella* Enteritidis (M).

3.2 Antimicrobial activity of probiotic yeasts and their effect on the inhibition of the adhesion of pathogens to Caco-2 cells

A desired characteristic for probiotic strains is to be able to compete for binding sites in the intestinal mucosa with pathogenic strains, inhibiting their adhesion and therefore reducing the probability of infection. The inhibition of the adhesion of pathogenic strains EPEC*,* *S.* Enteritidis and *L. monocytogenes* to Caco-2 cells was evaluated for the yeasts (*S. cerevisiae* CCMA 0731, *S. cerevisiae* CCMA 0732 and *P. kluyveri* CCMA 0615) previously selected by co-aggregation capacity and other tests performed earlier (data not shown) (Figure 2). To evaluate the competition for binding sites on the surface of Caco-2 cells, bacteria and yeasts were simultaneously inoculated into the plate wells. In addition, the exclusion capacity of yeasts toward pathogens was assessed first by inoculation and incubation of yeasts followed by a subsequent inoculation of bacteria. In general, the assays demonstrated that when yeasts and pathogens were co-incubated, the adhesion capacity of the pathogenic microorganisms to Caco-2 cells decreased, showing the antagonistic effect of yeasts on bacteria in the adhesion to intestinal cells.

The lowest percentages of adhesion inhibition (< 4%) were observed for the pathogenic bacteria EPEC independent of the yeast strain evaluated and type of assay (Figure 2). However, the *S. cerevisiae* CCMA 0731 and CCMA 0732 strains showed similar percentages ($p > 0.05$) of inhibition, around 2.3%, to the commercial strain *S. boulardii,* assessed by exclusion assays. Further, the *S. cerevisiae* CCMA 0731 and *P. kluyveri* CCMA 0615 strains showed higher ($p < 0.05$) percentages of EPEC inhibition (2.7% and 2.3%, respectively) than the *S. boulardii* strain (1.2%), as evaluated by competition assays. For the inhibition of *L. monocytogenes* adhesion to Caco-2 cells*,* the commercial strains showed the lowest ($p < 0.05$) percentage, 7.2%, evaluated by competition assay. The yeast *P. kluyveri* CCMA 0615 showed the highest ($p < 0.05$) percentage, 14%, observed by exclusion assays. On the other hand, *P. kluyveri* CCMA 0615 showed a low percentage of inhibition of *S.* Enteritidis adhesion to Caco-2 cells by both exclusion and competition assays (5% and 8.1%, respectively).

However, these values were similar ($p > 0.05$) to those obtained by *S. boulardii* strain. As observed, the response to the type of treatment employed (exclusion or competition) varied according to the yeast and pathogen strains.

The adhesion of pathogens to the surface of eukaryotic cells is mediated by macromolecules called adhesins (structures of the surface of the microorganism interacting with receptors specific to eukaryotic cells). A microorganism may express one or more adhesins, and that expression is regulated by environmental or host factors. Some pathogens have been reported to possess many different adhesins. Bacterial adhesins can be divided into two major groups: pili (fimbriae) and nonpilus adhesins (afimbrial adhesins) (Finlay and Falkow, 1997).

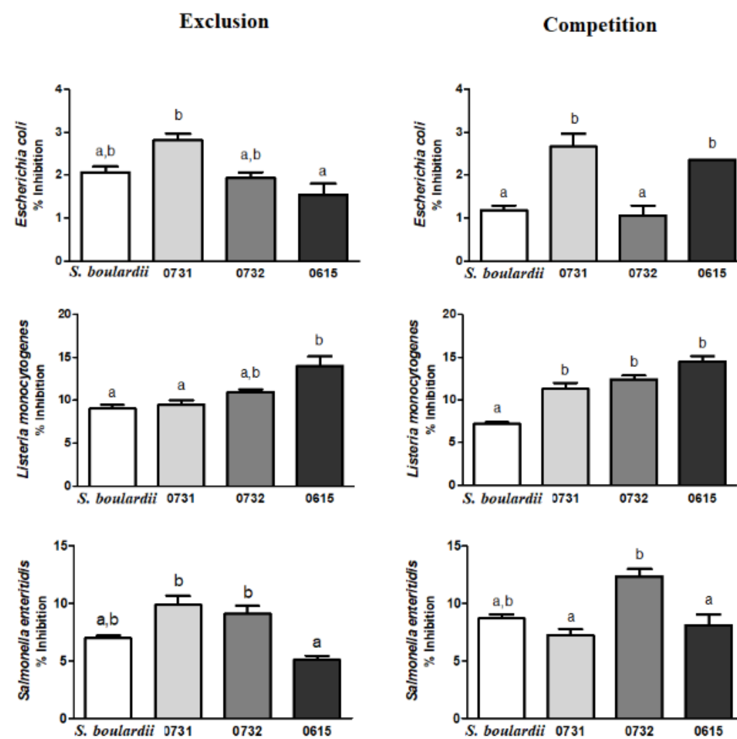


Fig. 2. Percentage of inhibition of foodborne pathogens EPEC, *Listeria monocytogenes* and *Salmonella* Enteritidis adhesion to Caco-2 cells evaluated by exclusion and competition assays inoculated with different yeast strains. Bars indicate standard deviations from the mean. Same letters over the bars indicate that the mean values did not differ significantly ($p < 0.05$) by Tukey.

The exclusion and competition assays using Caco-2 cells showed the percentage of inhibition of pathogens provided by the presence of yeast. Nevertheless, bacterial adhesion did not mean that the infection of intestinal cells occurred. Thus, the bacterial strains were stained, and confocal microscopy analyses were performed to estimate the infection level by pathogens in the presence or absence of yeasts (Figure 3).

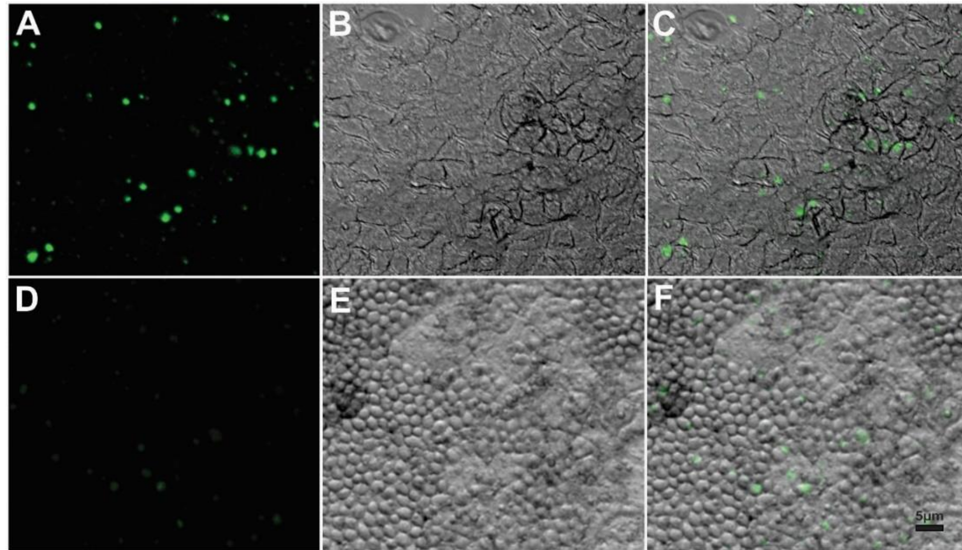
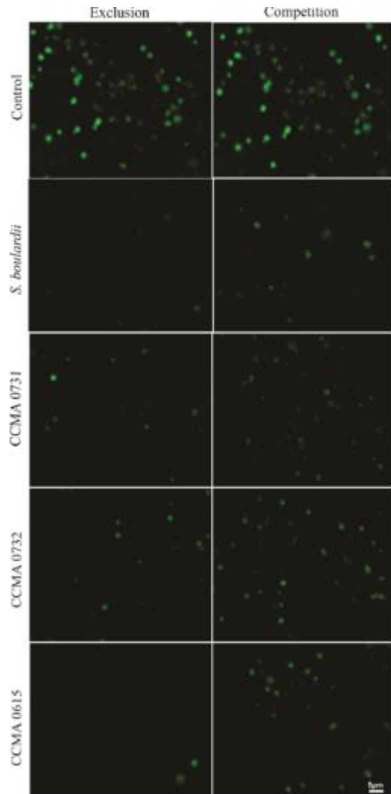


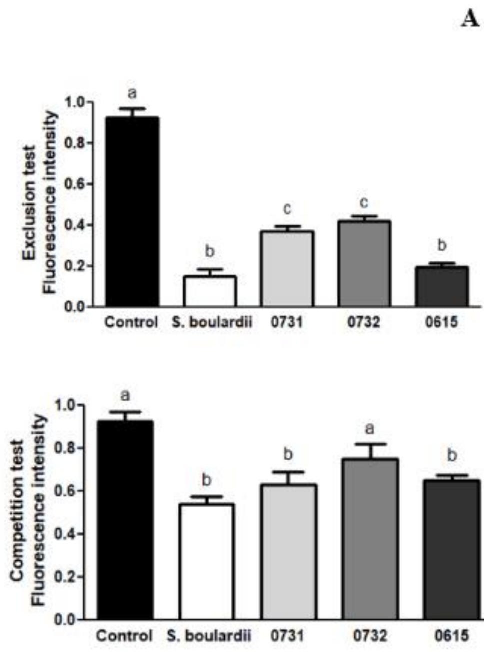
Fig. 3. Fluorescence micrographs taken using a laser confocal microscope, in which A, B and C represent the control without yeasts, and D, E and F represent treatment with yeasts. A and D: fluorescence of pathogenic bacteria labeled with *Syto9*. B and E: bright field images of Caco-2 cells and yeasts. C and F: overlap of fluorescence and bright field images.

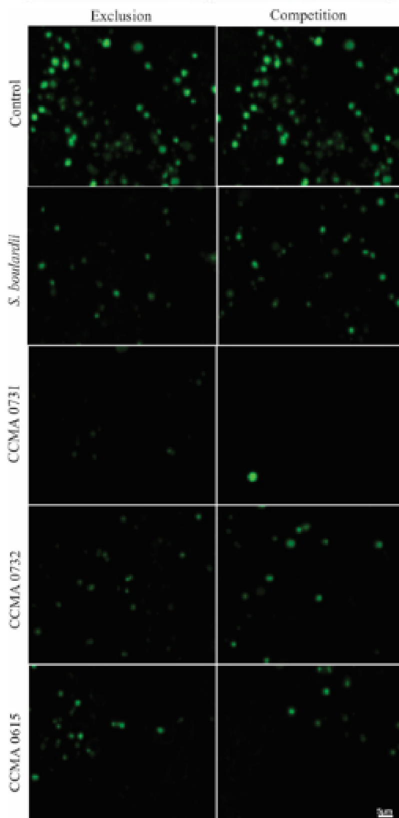
The results for estimated infection level based on fluorescence intensity are shown in Figure 4. All yeast strains were able to reduce the infection by the foodborne pathogens evaluated, except for *S.* Enteritidis, which the strains *S. cerevisiae* CCMA 0732 and *P. kluyveri* CCMA 0615 did not differ from the control (single bacteria inoculation). Regarding EPEC infection, the exclusion assay showed to be more effective for all yeast strains. *Pichia kluyveri* CCMA 0615 was able reduce significantly ($p < 0.05$) the infection of EPEC, similar ($p < 0.05$) to the commercial strain *S. boulardii*. On the other hand, the strain *S. cerevisiae* CCMA 0731 in addition to *P. kluyveri* CCMA 0615 also reduced ($p < 0.05$) EPEC infection similar to *S.*

boulardii for the competition assay. Further, *S. cerevisiae* CCMA 0731 reduced *L. monocytogenes* infection more than *S. boulardii* by competition assay. All yeasts reduced *L. monocytogenes* infection in a similar ($p > 0.05$) way for the exclusion treatment. The *S. cerevisiae* CCMA 0731 strains reduced *S.* Enteritidis infection more ($p < 0.05$) than *S. cerevisiae* CCMA 0732 and *P. kluyveri* CCMA 0615 and similar ($p > 0.05$) to the commercial yeast *S. boulardii* in both assays. According to Czerucka et al. (2000), the infection of T84 cells by *E. coli* in the presence of *S. boulardii* by exclusion assay was significantly reduced by 50%, similar to the result found in this present work, in which was observed a reduction of 50% or more of fluorescence intensity in the exclusion assays for EPEC, and *S.* Enteritidis and in the competition assay for *L. monocytogenes*, also varying among yeasts studied. According to the results, the better effect of ingesting probiotic yeasts as prevention (exclusion assays) or treatment (competition assays) for foodborne pathogens infection was strain-dependent. However, in general, both treatments showed positive effects when compared with the control and may indicate ingestion as prevention as well as a treatment of foodborne pathogens.



A





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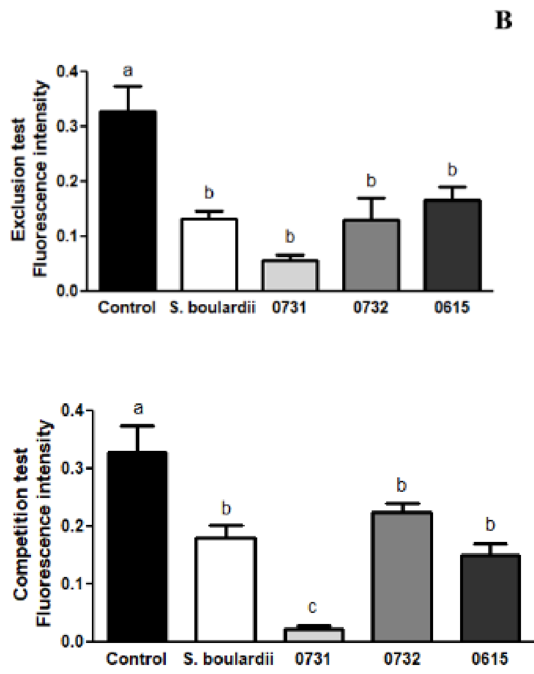

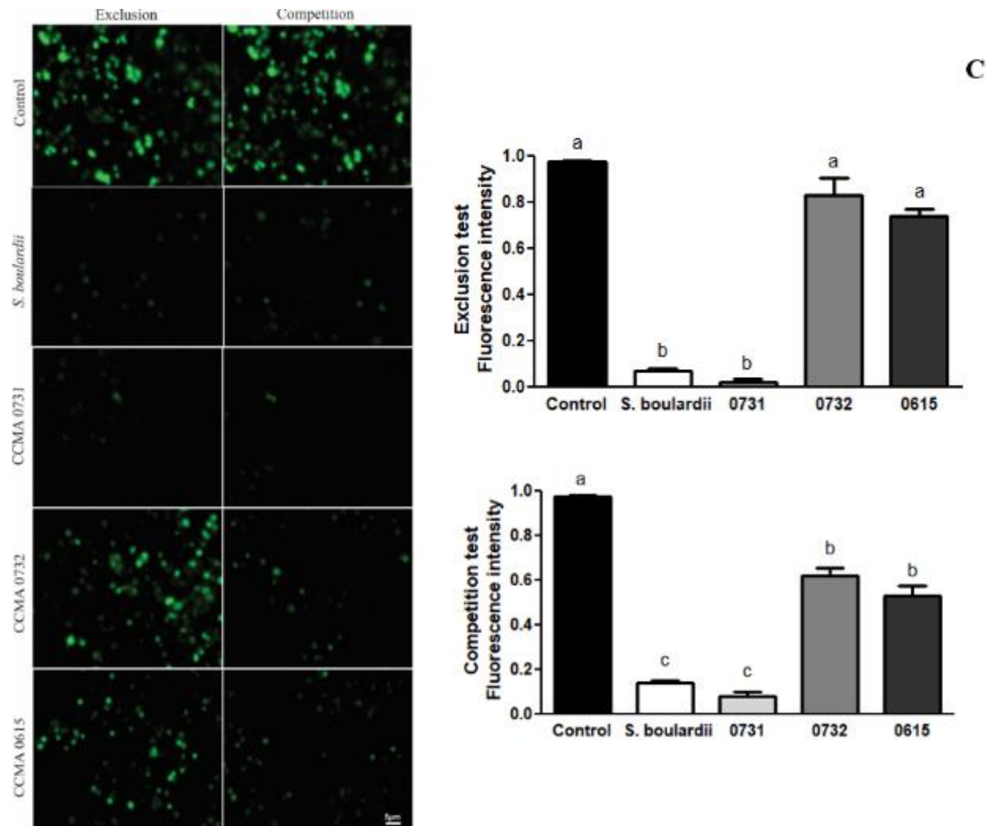

Fig. 4. Fluorescence intensity of foodborne pathogens EPEC (A), *Listeria monocytogenes* (B) and *Salmonella* Enteritidis (C) dyed by *Syto9* in the exclusion and competition assays inoculated with different yeasts strains, evaluated by laser confocal microscope. Same letters over the bars indicate that the mean values did not differ significantly ($p < 0.05$) by Tukey.

None of the tested yeasts showed antimicrobial activity toward the pathogens EPEC, *Salmonella* Enteritidis CDC 564 and *Listeria monocytogenes* ATCC 19117. The antagonism of yeasts against bacteria has not been often observed and described in the literature. Other authors have demonstrated that little inhibitory activity of yeasts against pathogenic microorganisms has been observed (Binetti et al., 2013; Silva et al., 2011). Martins et al. (2009) evaluated the antimicrobial activity of the probiotic bacteria *Bifidobacterium animalis*, *E. coli* and *Lactobacillus casei*, and the probiotic yeast *S. boulardii* and found that only *B. animalis* and *L. casei* showed antagonistic activities against the pathogens *S.* Typhimurium,

EPEC and *L. monocytogenes*. These findings reaffirm that the probiotic action of yeasts toward pathogens is associated with other mechanisms, such as co-aggregation, competition by binding sites on the cell surface and other, independent of the production of antimicrobial compounds. Regarding the mechanisms of inhibition of adhesion, in this study, a relationship between the yeast strains and the pathogenic agents can be observed by co-aggregation activity. This shows the importance of this mechanism in the prevention of a tissue colonization by pathogens. However, the cells receptors associated with the competition by binding sites on the cell surface should be further investigated.

4 Conclusion

The yeasts *Saccharomyces cerevisiae* CCMA 0731, *S. cerevisiae* CCMA 0732 and *Pichia kluyveri* CCMA 0615 isolated from fermented foods were able to inhibit the adhesion as well as the infection of Caco-2 cells by the foodborne pathogens EPEC, *Salmonella* Enteritidis and *Listeria monocytogenes*; however, the inhibitory effect was strain-dependent. Further, the exclusion and competition assays showed that the highest activity of the probiotic yeast ingestion as prevention or during the treatment of a foodborne infection was also strain-dependent, although both methods showed positive effects CCMA 0615 was more efficient against EPEC in the exclusion test, CCMA 0731 was more efficient in the competition test with *L. monocytogenes* and as efficient as the commercial strain in the exclusion and competition tests with *S*. Enteritidis.

It seems that the yeast strains compete with the evaluated pathogens by the same receptors and/or occupy their binding sites on the surface of the Caco-2 cells. Thus, these strains can be used as bio-therapeutic agents and as protective cultures in the attenuation of foodborne infection processes. However, more studies are needed to better understand the mechanism

and the cell receptors associated with microbial adhesion as well as to evaluate how microbial adhesion acts in response to the immune system in the host.

Acknowledgements

The authors thank CCMA and Prof Roberta H. Piccoli for providing the microbial strains and agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico do Brasil (CNPq), Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG), and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) for financial support.

The authors declare no conflicts of interest.

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ARTIGO 3- Combination of probiotic yeast and lactic acid bacteria as starter culture to produce maize-based beverages

Artigo publicado no periódicos Food Research International.

Abstract

Cereal-based fermented beverages are non-dairy products which are considered possible carriers for probiotic strains and alternatives for use by vegans and lactose-intolerant consumers. In the present work, the commercial probiotic, *Lactobacillus paracasei* LBC-81, was used singly and in co-culture with potential probiotic yeasts, *Saccharomyces cerevisiae* CCMA 0731, *S. cerevisiae* CCMA 0732, and *Pichia kluyveri* CCMA 0615, to ferment a maize-based substrate. All tested strains showed viability higher than 6 log CFU/mL, as recommended for food probiotic products, except for the yeast *P. kluyveri* which decreased during fermentation and storage time. A reduction in pH, from approximately 7 to 4, was observed. This decrease was due organic acid production, which did not affect the microbial viability. Lactic and acetic acids were the main organic acids produced during fermentation, and they decreased over 28 days of storage (< 0.5 and 0.1 g/L for lactic and acetic acids, respectively). Ethanol was detected in the *S. cerevisiae* assays; however, the content was < 5 g/L in a non-alcoholic beverage. Seventy volatile compounds were detected, including acids, alcohols, aldehydes, esters, ketones, and other compounds. Sensory analysis showed score of 5.93 – 4.57, respectively for appearance and taste. This is an important result, considering that the beverage had no flavoring additive and lacked a sweet taste. Therefore, probiotic beverages were successfully obtained by maize fermentation inoculated with co-culture of *S. cerevisiae* (CCMA 0731 or CCMA 0732) and *L. paracasei* LBC-81.

Keywords: Non-dairy beverages, probiotic beverage, *Lactobacillus paracasei*, *Saccharomyces cerevisiae, Pichia kluyveri,* volatile compounds.

1 Introduction

The increased demand for healthier foods over the last decade has prompted the development of novel products by the food industry, the so-called functional foods. Functional foods are foods or food ingredients that carry health benefits beyond traditional nutrient value (Ogunremi, Agrawal, & Sanni, 2015). Among these are the probiotic foods, which contain high populations of live probiotic microorganisms in their formulation. According to the Food and Agricultural Organization of the United Nations (FAO/WHO, 2002) and Hill et al. (2014), probiotics are live microorganisms that confer benefits to the host's health when administered in adequate amounts. Dairy products are the predominant probiotic carriers, probably due milk may aid in tolerating harsh gastro-intestinal condition, as its buffering action might protect probiotics in such conditions by reducing their direct exposure to harsh conditions (Ranadheera, Vidanarachchi, Rocha, Cruz, & Ajlouni, 2017). Furthermore, these products are well accepted by consumers worldwide. However, non-dairy products, especially cereal products, have gained global interest in response to major trends in food consumption, such as lactose intolerance, vegetarianism, veganism, and low-fat content foods (Ranadheera et al., 2017; Russo et al., 2017).

Plant-based materials, such as cassava (Freire, Ramos, & Schwan, 2015), maize, rice (Freire, Ramos, & Schwan, 2017), soy, peanuts, coconut water (Camargo Prado et al., 2015; Kantachote, Ratanaburee, Hayisama-ae, Sukhoom, & Nunkaew, 2017), soya extract, and quinoa (Bianchi, Rossi, Gomes, & Sivieri, 2015) have been shown to be potential substrates for developing novel functional fermented foods and beverages.

Indigenous plant-based fermented foods are important nutritional sources for many people, especially in developing countries in Africa, Asia, and Latin America. Ogi, kimchi, tempeh, fufu, cauim, calugi and pulque are some examples of the numerous fermented foods

produced from maize, sorghum, cabbage, soybean, cassava, rice, peanut, agave, and other substrates (Blandino, Al-Aseeri, Pandiella, Cantero, & Webb, 2003; Ramos & Schwan, 2017). Lactic acid bacteria (LAB) are an important group of bacteria often isolated and identified in these indigenous fermented foods. They produce lactic acid, decreasing the pH and making the food safe for consumption. However, in association with LAB, the yeasts have crucial importance in fermented foods. They contribute to organoleptic properties (Romano, Suzzi, Domizio, & Fatichenti, 1997) and to the improvement of human health with their probiotic properties (Moslehi-Jenabian, Pedersen, & Jespersen, 2010; Pedersen, Owusu-Kwarteng, Thorsen, & Jespersen, 2012). The co-cultivation of LAB and yeasts in fermented foods is well known. Yeasts contribute to LAB growth, and vice versa, since they provide some nutrients, such as amino acids and vitamins, to LAB, and their growth are favored by the low pH imposed by LAB metabolism (lactic acid production).

Saccharomyces cerevisiae, Pichia spp. and *Lactobacillus* spp. have been isolated from different spontaneous maize beverages such as chicha, calugi, pozol and boza (Blandino et al., 2003; Ramos & Schwan, 2017; Miguel et al., 2012). They have been considered as nontoxic, food-grade microorganisms, and most of them have a GRAS status (Generally Recognized as Safe) due their long history of safe use in foods. The co-culture of LAB and yeasts to ferment plant substrates in substitution to milk have been purposed in order to obtain non-dairy beverages as an alternative to the traditional dairy products with improved healthy and sensory characteristics (Freire et al., 2015; 2017; Santos et al., 2014).

Globally, cereals are very important crops, with maize, rice, and wheat being the predominant crops in terms of cultivated area and total production. In addition, these crops are important sources of nutrients, phytochemicals, and other bioactive compounds (Gionchetti et al., 2007). Maize is a widely used raw material in indigenous fermented beverage production; it contains about 72% starch, 10% protein, and 4% lipid and also provides many

of the essential minerals, vitamin B, and fiber (Ranum, Peña-Rosas, & Garcia-Casal, 2014). Several reports exist of functional cereal foods using lactobacilli as probiotic microorganisms, but of the use of yeasts with probiotic potential in these fermented foods little is known.

This work aimed to develop a novel, functional fermented beverage, using potentially probiotic yeasts isolated from Brazilian fermented foods to ferment maize substrate in association with the commercial probiotic strain *Lactobacillus paracasei* LBC-81. Microbial growth and survival during fermentation and storage were evaluated. Furthermore, data concerning the profiles of metabolites and volatile compounds, as well as the acceptance of the beverage by the consumers, were obtained.

2 Materials and methods

2.1 Microorganisms and culture conditions

The yeasts *Saccharomyces cerevisiae* CCMA 0732 and *S. cerevisiae* CCMA 0731 from caxiri, *Pichia kluyveri* CCMA 0615 from cocoa fermentation, all supplied to the Culture Collection of Agriculture Microbiology (CCMA) of the Federal University of Lavras (Brazil), and the commercial strain *Lactobacillus paracasei* LBC-81 (Danisco A/S, Copenhagen, Denmark) were used in this work. The yeast strains were previously selected based on probiotic technological characteristics such as survival at 37 °C, tolerance to low pH and bile salts, high hydrophobicity level, capacity of auto-aggregation, co-aggregation with pathogens and adhesion to Caco-2 cells (data not shown). The yeasts and LAB were stored at –80 °C in yeast extract-peptone-dextrose (YPD) broth at 10 g/L yeast extract (Merck, Darmstadt, Germany), 10 g/L peptone (Himedia, Mumbai, India), 20 g/L glucose (Merck, Darmstadt, Germany), and de Man, Rogosa and Sharpe (MRS) broth (Merck, Darmstadt, Germany), respectively, with 20% (v/v) glycerol. The yeast and LAB strains were reactivated by

streaking them onto YPD agar and MRS agar, respectively, and incubated for 24 h at 30 °C for yeast and 37 °C for LAB.

2.2 Fermentation medium for development of maize beverages

The medium for controlled fermentation assays was prepared based on the Brazilian indigenous beverage "calugi" (Miguel, Santos, Duarte, de Almeida, & Schwan, 2012), with some modifications. Approximately 550 g of dried maize (*Zea mays*) were used. First, the maize grains were soaked in water for 30 min and then macerated until a flour-like mixture was obtained. The resulting maize flour was mixed with 6 L of water and cooked for 1 h. Next, a proper starter culture (*L. paracasei* LBC-81) and yeast strain were added as described below; this was allowed to ferment at 30 °C for 24 h. The maize was purchased from the local market in Lavras, Minas Gerais, Brazil.

2.3 Fermentation assays and sampling

Five treatments were performed using different microorganisms: (1) *L. paracasei* LBC-81 + *S. cerevisiae* CCMA 0731; (2) *L. paracasei* LBC-81 + *S. cerevisiae* CCMA 0732; (3) *L. paracasei* LBC-81 + *P. kluyveri* CCMA 061; (4) *L. paracasei* LBC-81; and (5) control (without inoculation). The inoculum preparation was performed as described by Santos et al. (2014). The pre-grown LAB and yeast cells were washed twice with sterile peptone water at 0.1% w/v (Himedia, Mumbai, India) and recovered by centrifuging (6000 g) for 5 min at 20 °C. Microbial cells were inoculated in the substrate with a population of 6 log CFU/mL for the yeasts and 7 log CFU/mL for *L. paracasei* LBC-81. The fermentations were performed in 500 mL Erlenmeyer flasks, containing 400 mL of the substrates, at 37 °C for 24 h. All assays were performed in triplicate. After the fermentation time, the beverages were kept under refrigeration at 4 °C for 28d. Samples (5 mL) were taken at 0, 6, 12, and 24 h of fermentation

and at 7, 14, 21, and 28 d of refrigeration for subsequent analysis. The experiments were performed in three independent assays. For each repetition, two samples were taken at each time (duplicate).

2.4 Enumeration of microorganisms

Samples (1 mL) were taken from each fermentation flask. The total LAB, yeast, and Enterobacteriaceae populations were determined by plating in MRS agar (supplemented with 50 mg/L of nystatin), YPD agar (supplemented with 100 mg of chloramphenicol and 50 mg of chlortetracycline), and violet red bile glucose (VRBG) agar (Merck) media, respectively. Plates were incubated at 37 °C (LAB and Enterobacteriaceae) and at 30 °C (yeast) for 48 h, and the colony-forming units (CFU) were enumerated. The analyses were performed in triplicate.

2.5 Chemical analysis

2.5.1 Determination of pH

The pH levels of the fermenting beverage samples were measured using a pHmeter (Tecnal, Tec-3MT, São Paulo, Brazil).

2.5.2 Substrate and metabolite analysis by high-performance liquid chromatography (HPLC)

The analyses of organic acids, alcohols, and carbohydrates were carried out using an HPLC (Model LC-10Ai; Shimadzu Corp., Tokyo, Japan), equipped with a dual detection system consisting of a UV–vis detector (SPD-10Ai; Shimadzu) and a refractive index detector (RID-10Ai; Shimadzu). A Shimadzu ion exclusion column (Shim-pack SCR-101H, 7.9 mm × 30 cm) was used for alcohols, carbohydrates determination (30 °C) and organic acids (50 °C) (Duarte et al., 2010). The compounds were identified based on the retention time of standards,

and their concentrations were determined using the external calibration method. All samples were examined in duplicate.

2.5.3 Volatile compound extraction and gas chromatography with mass spectrometer (GC-MS) analysis

The volatile compounds of the beverage samples were extracted using the solid-phase micro-extraction technique in the headspace (SPME-HS), as described by Menezes et al., 2016, with minor modifications. Two mL of the samples were placed in a 15 mL sample vial. A 50 / 30 µm divinylbenzene /carboxene / polydimethylsiloxane (DVB / CAR / PDMS) fiber provided by Supelco (Bellefonte, PA, USA) was used to extract the volatile compounds. This fiber was balanced for 15 min at 60 °C and then exposed to the samples in the 15 mL vials for 30 min at the same temperature.

The volatile compounds were analyzed by gas chromatography–mass spectrometry (GC–MS) (Model GCMS-QP2010SE; Shimadzu, Tokyo, Japan) equipped with a Carbo - wax column (30 m × 0.25 mm id × 0.25 µm film thickness). The oven temperature was set at 40 °C for 5 min, increased until it reached 200 °C (at a rate of 10 °C/min), and finally maintained at this temperature for 30 min. The carrier gas was high purity helium, at 0.7 mL/min. Splitless injection was used. The selective mass detector was a quadrupole, with an electronic impact ionization system at 70 eV and at 260 °C. Volatile compounds were tentatively identified using GC/MS Solution ver. 2.6 software. Linear retention indices relative to a mixture of n-alkanes were calculated according to the Kovats retention index (KI). Volatile compounds were tentatively identified by probability-based matching of their mass spectra with those obtained from the NIST 11 GC Method/Retention Index Database and by matching the KI of the compounds with values from the literature.

2.6 Sensory analysis

Sensory analyses of the beverages were performed using a consumer acceptance test according to the hedonic scale of nine categories ranging from dislike extremely (1) to like extremely (9) (Stone and Sidel, 2004). Consumers evaluated the samples for appearance, color, aroma, taste, texture, and general acceptability.

The tests were performed in closed cabins with white illumination at the Sensory Analysis Laboratory, Federal University of Lavras (Lavras, Minas Gerais, Brazil). The samples were labeled with three random digits on a white surface. These samples had a monadic form and followed a balanced order of presentation (Wakeling & MacFie, 1995). Sixty untrained panelists were selected based on their consumption of non-alcoholic fermented beverages; they ranged from 18 and 60 years of age and were students and workers from the Federal University of Lavras. Randomized 15 mL samples were served in clear 50 mL glasses at between 4–10 °C. The consumers rinsed their mouths with water between tastings.

2.7 Statistical analysis

The data were subject to analysis of variance (ANOVA), and the statistical analyses were performed using Sisvar 5.3 software (Ferreira, 2014). For sensory analysis SensoMaker 1.7 software was used and differences in values were considered significant when the *P*-value was < 0.05. Volatile compounds were analyzed by principal component analysis (PCA) using SensoMaker 1.7 software (Nunes & Pinheiro, 2012).

3 Results and discussion

3.1 Microbial growth and acidification profiles during fermentation beverages

The yeast strains *S. cerevisae* CCMA 0731, *S. cerevisiae* CCMA 0732, and *P. kluyveri* CCMA 0615, previously characterized as probiotic (data not shown), were separately

employed in combination with the commercial probiotic bacteria *L. paracasei* LBC-81 to ferment maize substrate. The substrate preparation was based on the Brazilian indigenous beverage calugi (Miguel et al., 2012), however, the starter culture were choice based on their probiotic description.

Fig. 1 shows the microbial growth and pH during 24 h of fermentation for the different assays. The LAB population showed a similar ($p > 0.05$) dynamic when inoculated in a single culture or mixed with *S. cerevisiae* strains CCMA 0731 and CCMA 0732, keeping its population around 7.6 log CFU/mL at 24 h (Fig. 1A, B, and C). When the LAB was cultured in association with *P. kluyveri*, the bacteria population decreased from 7.9 to 7.1 log CFU/mL during 24 h (Fig. 1D). However, all assays showed a LAB population at the end of fermentation above the threshold recommended for probiotic microorganisms (Brasil, 2002).

Regarding the dynamic of the yeast population, *S. cerevisiae* CCMA 0731 and 0732 showed similar ($p > 0.05$) behavior over the first 12 h of fermentation, which was different from *P. kluyveri* behavior. At 24 h of fermentation, all assays showed yeast populations which were significantly ($p < 0.05$) different. *S. cerevisiae* CCMA 0731 remained almost constant during the 24 h of fermentation (6.7 log CFU/mL), while *S. cerevisiae* CCMA 0732 showed a slight growth reaching 7.0 log CFU/mL at the end of fermentation (Fig. 1B and C). On the other hand, *P. kluyveri* decreased in population from 6.1 log CFU/mL to 5.7 log CFU/mL (Fig. 1D), and, therefore, did not maintain the recommended viable cells for probiotic beverages. It is known that LAB may positively affect the yeast growth in fermented foods by acidification of the environment, and the yeast may provide growth factors such as vitamin and amino acids to LAB (Nout & Sarkar, 1999). However, this was not the case for the combination of *L. paracasei* LBC-81 and *P. kluyveri* CCMA 0615 in the present study of fermenting maize substrate. This assay showed the highest pH at the end of fermentation (around pH 5.0) as compared to the assays inoculated with *S. cerevisiae* strains (around pH

4.0). For all assays, the pH was rapidly reduced in the first 6 h of fermentation (Fig. 1). The pH reduction demonstrates the action of microorganisms during fermentation by the production of organic acids, such as lactic and acetic acids. *L. paracasei* is a facultative heterofermentative bacteria which may produce acetic acid in addition to lactic acid. The pH reduction is important as it affects shelf life and prevents food contamination (Rathore, Salmerón, & Pandiella, 2012). It was observed that, in the assay with a single culture of LAB, the pH at the end of maize fermentation was higher (5.1) than those with the mixed cultures of LAB and yeasts (Fig. 1).

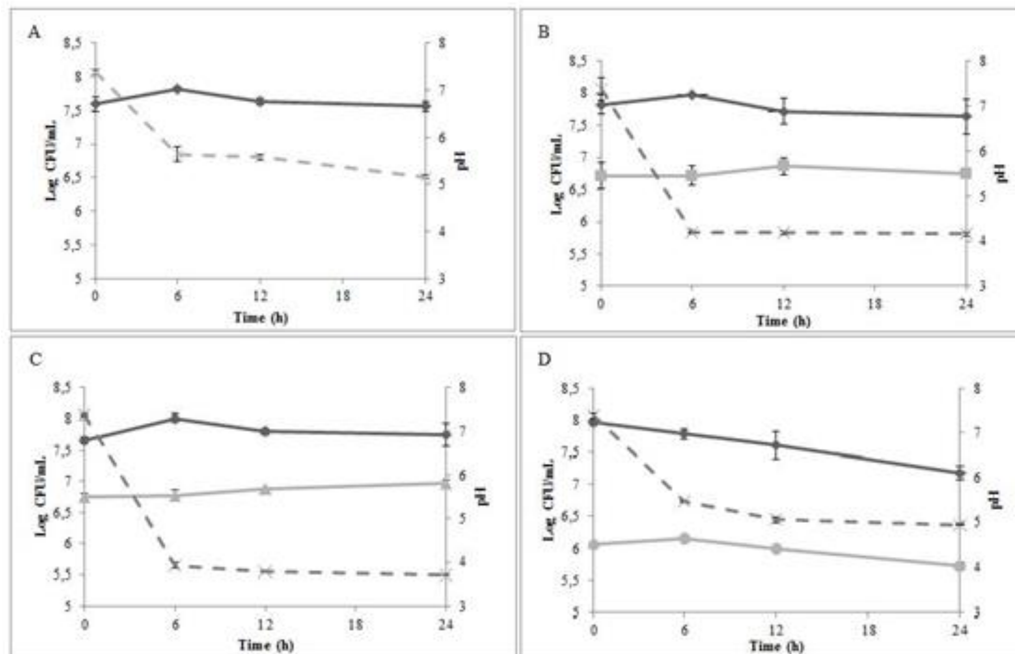

Fig. 1. Microbial populations of (♦) *L. paracasei* LBC-81 **(A)**; (♦) *L. paracasei* LBC-81 + (■) *Saccharomyces cerevisiae* CCMA 0731 **(B)**; (♦) *L. paracasei* LBC-81 + (▲) *Saccharomyces cerevisiae* CCMA 0732 **(C)**; (♦) *L. paracasei* LBC-81 + (●) *Pichia klyvery* CCMA 0615**(D)**; and (×) pH during 24 h of maize fermentation. The assays were performed in triplicate and analyzed by Scott-Knott test ($p<0.05$).

The combination of LAB and yeasts during fermentation of vegetable-based beverages is well documented (Ai, Li, Su, & Meng, 2015; Freire, Ramos, da Costa Souza, Cardoso, & Schwan, 2017; Freire et al., 2015; Miguel et al., 2012; Ramos et al., 2010; Santos, Libeck, & Schwan, 2014). Yeasts have been successfully employed as starter cultures for non-dairy beverage elaboration, producing compounds which confer a pleasant aroma and flavor (Freire et al., 2015; Santos et al., 2014). Freire et al. (2015) performed fermentations of cassava using single and co-cultures of LAB and yeast *(S. cerevisiae, Pichia caribbica,* and *Torulaspora delbrueckii*) as starter cultures, and they observed that ethanol concentration was approximately three times lower (around 0.15% w/v) in the co-culture assays. The authors suggested that this may have been due to competition for nutrients between yeast and LAB and, consequently, the lack of production of fermentable sugars for ethanol by the yeasts. This data is very relevant to the production of non-alcoholic beverages using fermenting yeasts.

3.2 Probiotic viability during storage period

The viability of LAB and yeast cells was evaluated after 7, 14, 21, and 28 days of storage at 4 °C, and the results are shown in Fig. 2. According to the obtained data, *L. paracasei* viability was significantly higher ($p < 0.05$) in the assays with inoculated with both *S. cerevisiae* CCMA 0731 and CCMA 0732, when compared to the assays inoculated with *P. kluyveri* CCMA 0615 and single culture, during all storage time. Regarding yeast cells, *S. cerevisae* CCMA 0731 and CCMA 0732 showed higher ($p < 0.05$) viability than *P. kluyveri* CCMA 0615 during the 28 days of storage.

All tested strains maintained their population almost constantly during the storage period. The *L. paracasei* population showed a population ranging from 7.2 log CFU/mL (assays in single culture and co-cultivation with *P. kluyveri*) to 7.7 log CFU/mL (assays in co-cultivation with both *S. cerevisiae* strains). Values of viable cells were still above those

recommended for probiotic products. The legislation of many countries considers that the bacterial population of foods must be viable during storage, but the minimum levels established for an effective quantification may vary from 10^6 to 10^8 CFU/g, depending on local regulations. The minimum amount of viable probiotic bacteria in milk products should be between 6 and 7 log CFU/g according to European legislation (Grand, Küffer, & Baumgartner, 2003), and values lower than 10^8 CFU/g may be acceptable by Brazilian legislation since its effectiveness is proven (Brasil, 2002). Regarding the viable cells of yeasts, only *P. kluyveri* showed a population (5.4 log CFU/mL) lower than that recommended by legislation. *S. cerevisiae* CCMA 0731 and *S. cerevisiae* CCMA 0732 showed 6.9 and 6.7 log CFU/mL, respectively, after 28 days of storage. Several studies have been performed with the purpose of developing non-dairy functional foods by evaluating their LAB population (Costa, Soares Júnior, Rosa, Caliari, & Pimentel, 2017; Freire, Ramos, da Costa Souza, et al., 2017; Santos et al., 2014); however, there are no data about fermented foods inoculated with probiotic yeast. Freire et al. (2017), developed maize and rice beverages by inoculating the probiotic *Lactobacillus acidophilus* LACA 4 and *L. plantarum* CCMA 0743, and the amylolitic yeast *Torulaspora delbrueckii* CCMA 0235 as the starter culture and observed that the probiotic strains remained viable during 28 days of storage. On the other hand, Costa et al. (2017) observed a decrease from 10^6 to 10^5 and 10^4 CFU/g after 28 days of storage for *L. acidophilus* and *Bifidobacterium* spp. cells, respectively, in beverages produced from soya and rice by-products. The viability of cells during a storage period may be affected by various factors, such as strains, substrate, and temperature.

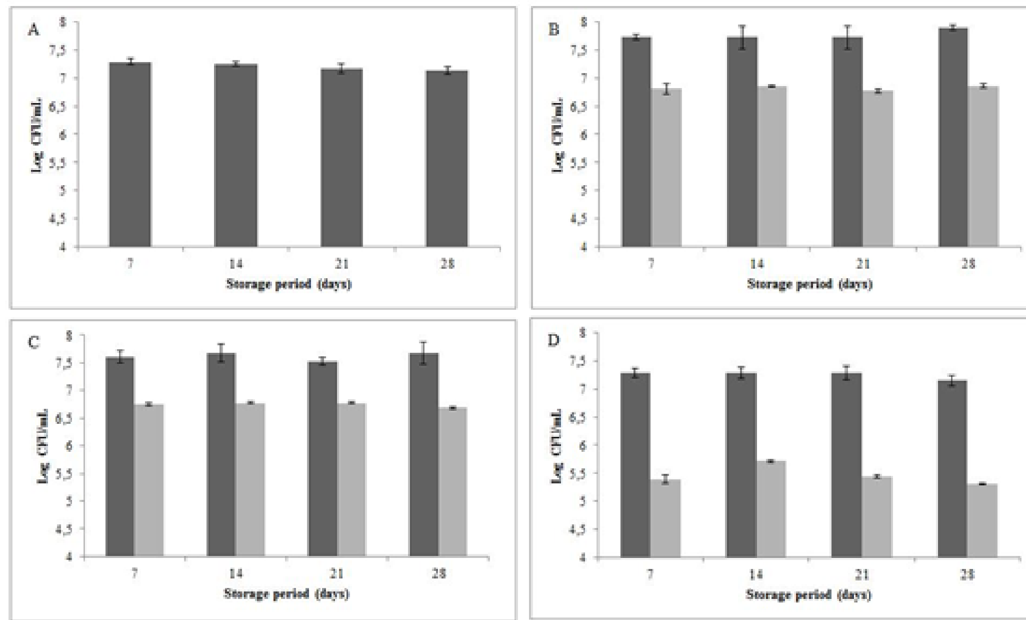

Fig. 2. Microbial viability of *L. paracasei* LBC-81 **(A);** *L. paracasei* LBC-81 + *S. cerevisiae* CCMA 0731 **(B)**; *L. paracasei* LBC-81 + *S. cerevisiae* CCMA 0732 **(C);** and *L. paracasei* LBC-81 + *P. klyvery* CCMA 0615 **(D)** during 28 days of storage at 4°C. Dark grey corresponds to *L. paracasei* viability and light grey corresponds to yeast viability in the different assays. The assays were performed in triplicate and analyzed by Scott-Knott test ($p<0.05$).

No ingredient that could enhance the survival of the microorganisms was added in the present study. Freire et al. (2017) observed that the fructooligosaccharides addition to maize and rice substrates was important to maintain a viable population of probiotic and yeast *T. delbrueckii* during storage. It could be an alternative used to improve *P. kluyveri* CCMA 0615 viability during

3.3 Chemical parameters

The results of carbohydrate, organic acid, and alcohol analysis evaluated by HPLC are shown in Table 1. The compounds were quantified in the substrate (0 h) and final (24 h)

fermentation, and at the final storage period (28 days). The carbohydrates, sucrose, fructose, and glucose were consumed during fermentation and remained almost constant during the storage time. Sucrose was the main carbohydrate detected in the substrate (0 h), as was also observed by Freire et al. (2017), using maize and rice as a substrate and *L. acidophilus* LACA 4 (Danisco A/S, Copenhagen, Denmark) as a probiotic strain. A residual concentration remained at the end (24 h) of fermentation and after 28 days of storage in all assays. As expected, no modifications were observed in the control assay for all chemical parameters since it was not inoculated with microorganisms.

Lactic acid was the main metabolite produced during fermentation for all assays. However, the assays with single LAB and those containing LAB and *S. cerevisiae* strains showed higher concentrations (> 0.5 g/L) than the assay with LAB and *P. kluyveri* (0.28 g/L). This may be related to the decrease of LAB population in the assay containing *P. kluyveri*, as observed in Fig. 1. Acetic acid was also produced during fermentation showing concentrations ranging from 0.1 to 0.2 g/L among the assays. *L. paracasei* is a facultative heterofermentative LAB and may produce acetic acid in addition to lactic acid both of which are related to pH decrease after 24 h of fermentation. Lactic and acetic acids showed a slight decrease after 28 days of storage (< 0.5 and 0.1 g/L for lactic and acetic acids, respectively), except in the assay inoculated with a single culture of LAB which showed acetic acid concentration of 0.34 g/L. The decrease of acids, mainly acetic acid, may be a positive factor, since this acid may provide an off-flavor in high concentrations. Citric acid was consumed during fermentation and was not detected after fermentation and the storage period as also observed by Freire, Ramos & Schwan (2017), while succinic and malic acids maintained almost constant levels (around 0.3 g/L) (Table 1). Previous studies with co-cultivation of LAB and yeasts during plant-based fermentations demonstrated the important effects of organic acids and volatile compounds on the flavor and aroma profile of the final product

(Freire et al., 2015; Freire et al., 2017; Salim-ur-Rehman, Paterson, & Piggott, 2006). Freire et al. (2015) suggested an interaction between LAB and yeast for tartaric acid production which were only detected in the assays with co-culture of LAB and yeasts.

Regarding alcohol concentrations, ethanol was only detected in the assays inoculated with LAB and *S. cerevisiae* strains at 24 h of fermentation. The concentrations were 0.20 g/L and 0.07 g/L for CCMA 0731 and CCMA 0732 strains, respectively. These values were below 5 g/L, which is the maximum permissible concentration to be considered in non-alcoholic beverages (Brasil, 2009). At 28 days of storage, ethanol was not detected for the assay with CCMA 0731 and decreased to 0.02 g/L for the assay with CCMA 0732. Low ethanol concentrations have been registered by other authors studying co-cultivation of yeast and bacteria in cereal substrates (Ai et al., 2015; Freire, Ramos, da Costa Souza, et al., 2017; Santos et al., 2014). Another alcohol found in the fermentations was glycerol. This compound was produced during all fermentation; however, the assays inoculated with *S. cerevisiae* strains showed higher concentrations (around 0.2 g/L) than the assays containing single LAB and LAB with *P. kluyveri* (around 0.1 g/L) at 24 h of fermentation. After the storage period, there was a slight decrease in the concentrations for all assays, and the concentration in the final products did not exceeded 0.2 g/L. Glycerol is produced by yeasts, mainly by the *S. cerevisiae* strains. Its production increases under stressful conditions, as in the case of osmotic stress, during competition for nutrients, or when there are toxic metabolites in the environment, factors generally found at the end of fermentation (Aslankoohi, Rezaei, Vervoort, Courtin, & Verstrepen, 2015). Glycerol may have a positive impact on the beverages due how it contributes to the body and fullness of the product, and it is suspected to contribute better to non- and low-alcoholic beverages, considering the organoleptic impacts described by Zhao, Procopio, & Becker (2015).

Table 1. Concentration of carbohydrates, organic acids and alcohols in maize based beverages fermented with different starter cultures.

Fermentation (hour)	Samples	Compound concentration (g/L)									
		Sucrose	**Fructose**	**Glucose**	**Citric acid**	**Malic acid**	**Succinic acid**	**Lactic acid**	**Acetic acid**	**Etanol**	**Glicerol**
0	Substrate	3.19 ± 0.02	0.02 ± 0.01	0.01 ± 0.01	0.06 ± 0.03	0.05 ± 0.01	0.01 ± 0.00	ND	ND	ND	ND
24	Control	3.23 ± 0.07^{aA}	0.03 ± 0.001^{aB}	0.02 ± 0.001^{aA}	0.12 ± 0.01^{aA}	0.06 ± 0.02^{aA}	0.02 ± 0.001^{aA}	ND^{cA}	ND^{cA}	ND^{cA}	ND^{cA}
	L. paracasei LBC-81	2.59 ± 0.52^{aA}	0.01 ± 0.001^{bA}	0.01 ± 0.001^{abA}	ND^{bA}	0.03 ± 0.00^{bA}	0.02 ± 0.01^{aA}	0.55 ± 0.02^{aA}	0.22 ± 0.01^{aA}	ND^{cA}	0.11 ± 0.01^{bA}
	L. paracasei LBC-81 + CCMA 0731	2,39 ± 0,13^{aA}	ND^{cA}	ND^{bA}	ND^{bA}	0.04 ± 0.00^{abA}	0.03 ± 0.00^{aA}	0.50 ± 0.06^{aA}	0.10 ± 0.02^{bA}	0.20 ± 0.03^{aA}	0.21 ± 0.01^{aA}
	L. paracasei LBC-81 + CCMA 0732	ND^{bA}	ND^{cA}	ND^{bA}	ND^{bA}	0.04 ± 0.00^{abA}	0.03 ± 0.00^{aA}	0.65 ± 0.05^{aA}	0.19 ± 0.02^{aA}	0.07 ± 0.02^{bA}	0.25 ± 0.02^{aA}
	L. paracasei LBC-81 + CCMA 0615	2.90 ± 0.11^{aA}	0.01 ± 0.01^{bA}	ND^{bA}	ND^{bA}	0.01 ± 0.00^{bA}	0.09 ± 0.05^{aA}	0.28 ± 0.03^{bA}	0.17 ± 0.01^{aA}	ND^{cA}	0.12 ± 0.02^{bA}
Storage (days)											
28	Control	3.20 ± 0.02^{aA}	0.02 ± 0.002^{aB}	0.02 ± 0.00^{aA}	ND^{aB}	0.08 ± 0.06^{aA}	0.04 ± 0.001^{aB}	ND^{bA}	ND^{bA}	ND^{bA}	ND^{dA}
	L. paracasei LBC-81	2.53 ± 0.001^{bcA}	0.01 ± 0.001^{abA}	0.01 ± 0.001^{aA}	ND^{aA}	0.05 ± 0.00^{aB}	0.03 ± 0.002^{abB}	0.34 ± 0.10^{aA}	0.34 ± 0.02^{aB}	ND^{bA}	0.09 ± 0.001^{cA}
	L. paracasei LBC-81 + CCMA 0731	2.40 ± 0.13^{bcA}	ND^{bA}	ND^{bA}	ND^{aA}	0.03 ± 0.02^{aA}	0.02 ± 0.004^{abA}	0.31 ± 0.02^{aA}	0.06 ± 0.02^{aA}	ND^{bB}	0.12 ± 0.01^{bB}
	L. paracasei LBC-81 + CCMA 0732	2.12 ± 0.10^{bB}	ND^{bA}	ND^{bA}	ND^{aA}	ND^{aB}	0.05 ± 0.01^{aB}	0.50 ± 0.01^{aA}	ND^{bB}	0.02 ± 0.001^{aA}	0.20 ± 0.002^{aA}
	L. paracasei LBC-81 + CCMA 0615	2.75 ± 0.11^{cA}	0.01 ± 0.006^{abA}	0.01 ± 0.005^{aB}	ND^{aA}	ND^{aB}	0.005 ± 0.00^{bA}	0.03 ± 0.02^{bB}	ND^{bB}	ND^{bA}	0.11 ± 0.005^{bB}

ND-not detect

Mean ± SD. Values followed by same lower-case letters at same column did not significantly differ in each time by Tukey test. And values followed by same capital letters at same column did not significantly differ at 24 h and 28 days by Tukey test

A total of 70 compounds were identified by GC-MS, considering all fermentations assays. These compounds included acids, alcohols, aldehydes, esters, ketones, and others (Table 3). Alcohols constituted the major group of volatile compounds (25 compounds) detected, followed by acids (8), ketones (7), aldehydes (6), and esters (4). The co-cultivation of LAB and yeasts showed a higher number of alcohol compounds than the assay with only the LAB strain. Yeasts are important alcohol producers that, together with their derived esters, have unique flavor properties (Longo & Sanromán, 2006). Some alcohols, which are related to pleasant flavor characteristics, such as 1-hexanol (fruity odor and aromatic flavor), 1-heptanol (fragrant, woody, heavy, oily, faint, aromatic, fatty odor, and a pungent, spicy taste), 1-octanol (fresh, orange-rose odor which is quite sweet), 1-nonanol (fresh, orange, rose), phenylethyl alcohol (rose-like odor), and 2-methyl-1-butanol (cooked, roasted aroma with fruity or alcoholic undernotes), were present at end (24 h) of fermentation. Also, 2-methyl-1-butanol, phenylethyl alcohol, 1-hexanol, and 1-nonanol remained detectable after 28 days in all assays inoculated with yeasts. Muyanja, Narvhus, & Langsrud (2012) evaluated volatile compounds during spontaneous and controlled (inoculated with LAB) fermentation of Bushera and observed that the concentrations of 2-methyl-1-butanol, 3-metil-1-butanol, and 2-metil-1-propanol in the spontaneous fermentations were higher than those inoculated with LAB. These authors suggested that yeast presence in the spontaneous process may favor the production of these alcohols during Bushera fermentation. The alcohols 2-metil-1-butanol and 3-metil-1-butanol were detected in the present study in the assays inoculated with yeast. The compound 3-metil-1-butanol was detected in the assay inoculated with *P. kluyveri* and *S. cerevisiae* CCMA 0731. Amyl alcohols, such as 3-metil-1-butanol and 2-metil-1-butanol, are formed during the fermentation by

deamination and decarboxylation of isoleucine and leucine, respectively (Boulton et al., 1996). Annan, Sefa-Dedeh, Plahar, & Jakobsen (2003), studying maize fermented products, detected higher concentrations of the alcohols 1-propanol, 2-metil-1-propanol, and 3-metil-butanol in the fermentations with *S. cerevisiae*, while higher amounts of phenylethyl alcohol were found in the fermentations with *Candida krusei.*

Phenylethyl alcohol is one of the most common alcohols produced by yeasts, giving off a rose aroma (Longo & Sanromán, 2006) and it was detected in the present study in all beverages inoculated with yeast, at 24 h of fermentation and after the 28 days of storage.

Among the identified ketones, 6-methyl- 5-hepten-2-one, and isophorone are known for their aroma of fresh fruit and a slight taste of mint, respectively.

Table 2 Volatile compounds identified by GC–MS analysis at the beginning (0 h), and final fermentation time (24 h) and during storage period (28 days) of different assays.

				Assay				
Chemical class	**PCA**	**Volatile coumpounds**	**Sensory perception**	**Substrate**	***Lactobacillus paracasei***	**Lac + CCMA 0731**	**Lac + CCMA 0732**	**Lac + CCMA 0615**
	1	2-ethyl-1-Hexanol	-	-	F_F, S	F_F, S	S	F_F
	2	1-Dodecanol	Characteristic fatty odor; fatty, waxy flavor	P	F_F, S	F_F, S	F_F, S	F_F, S
	3	2-(dodecyloxy)-ethanol	-	P	S	S	F_F	-
	4	2,4-bis(1,1-dimethylethyl)-Phenol	-	P	F_F, S	F_F, S	F_F, S	S
	5	1-Decanol	Floral odor resembling orange flowers and a slight, characteristic fatty taste	-	F_F, S	F_F, S	F_F, S	F_F, S
	6	1-Hexanol	Flavoring ingredient: fruity odor and aromatic flavor	-	F_F	F_F, S	F_F, S	F_F, S
	7	1-Heptanol	Fragrant, woody, heavy, oily, faint, aromatic, fatty odor, and a pungent, spicy taste	-	F_F	S	-	F_F
	8	4-ethyl-1,3-Benzenediol	-	-	F_F	S	S	S
	9	1-Octanol	Fresh, orange-rose odor, that is quite sweet	P	F_F, S	F_F, S	F_F, S	F_F
	10	2-Nonen-1-ol	-	-	F_F	-	S	-
	11	1-Hexadecanol	Faint waxy, nearly odorless	P	F_F, S	S	F_F	-
Alcohol	12	1-Nonanol	Fresh, orange, rose	P	F_F, S	S	F_F, S	F_F, S
	39	n-Heptadecanol-1	-	P	F_F	-	-	-
	13	Phenylethyl Alcohol	Rose-like odor	P	-	F_F, S	F_F, S	F_F, S

	51	n-Pentadecanol	-	-	-	F_F	-	-
	52	n-Nonadecanol-1	-	-	-	F_F	-	-
	14	2-methyl-1-butanol,	Cooked, roasted aroma with fruity or alcoholic undernotes	-	-	F_F, S	F_F, S	F_F, S
	15	Ethanol	-	-	-	F_F, S	F_F, S	F_F, S
	16	3-methyl- 1-Butanol	Fuel oil, whiskey-characteristic, pungent odor	-	-	F_F, S	-	S
	17	1-Tetradecanol	Weak oily fatty	-	-	S	F_F	-
	62	Cyclodecanol	-	-	-	S	S	-
	54	(Z)- 3-Nonen-1-ol	-	-	-	-	F_F	-
	18	1-Pentanol	Fusel-like sweet and pleasant odor and burning taste	-	-	-	S	F_F
	55	2-Propyl-1-pentanol	-	-	-	-	S	-
	56	(Z)-3-Decen-1-ol	-	-	-	S	-	-
	-	Hexanal	Flavoring ingredient. Fatty, green, grassy, powerful, penetrating characteristic fruity odor and taste	P	-	-	-	-
	40	(Z)-2-Heptenal	Flavoring ingredient : green, fatty odor	-	-	-	F_F	-
Aldehyde	53	Nonanal	Strong, soap-like, metallic	P	F_F	-	-	-
	63	(E)- 2-decenal	-	P	S	-	-	-
	19	2,4-dimethyl-Benzaldehyde	-	P	F_F, S	F_F, S	F_F, S	F_F
	57	1,4-Benzenedicarboxaldehyde	-	-	-	S	-	-
	20	(Z)-6,10-dimethyl, 5,9-undecadien-2-one	Fatty, somewhat metallic flavor	-	F_F	S	S	-
	21	6-methyl- 5-hepten-2-one	Green, fresh fruity flavor	P	F_F, S	F_F, S	F_F, S	F_F, S

Ketone	22	Isophorone	Slight minty odor	P	F_F, S	F_F, S	F_F, S	F_F, S
	-	2-methyl- 3-Octanone	-	P	-	-	-	-
	41	3,5-Dimethyl-2-furyl methyl ketone	-	-	-	F_F	F_F	-
	23	1-(2,4-Dimethyl-furan-3-yl)-ethanone	-	-	-	F_F	F_F	S
	58	2(3H)-Furanone, (Z)-dihydro-5-(2-octenyl)-2(3H)-furanone	-	-	-	S	S	-
Acid	24	Acetic acid	Pungent, stinging sour	P	F_F, S	F_F, S	F_F, S	F_F
	42	2-hydroxy-benzoic acid	-	P	F_F	F_F	F_F	-
	-	Tetradecanoic acid	Very faint, waxy-oily; nearly odorless	P	-	-	-	-
	-	n-Hexadecanoic acid	Virtually odorless, bland taste	P	-	-	-	-
	25	3-methyl- butanoic acid	Fruitlike (apple, cherry) odor	-	F_F, S	F_F	-	-
	43	Dodecanoic acid	Fatty, unpleasant, rancid odor	-	F_F	F_F	F_F	-
	50	Hexanoic acid	Fatty-rancid odor, acrid-acid	-	F_F	F_F	F_F	F_F
	44	n-Decanoic acid		-	F_F	F_F	F_F	-
Ester	26	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	-	P	F_F, S	F_F, S	F_F, S	F_F, S
	45	Formic acid, heptyl ester	-	-	-	-	F_F	-
	46	Homosalate	-	-	-	-	-	F_F
	27	3-methyl, 1-butanol acetate	-	-	-	-	-	F_F, S
	28	decamethyl-cyclopentasiloxane,	-	P	F_F, S	F_F, S	F_F, S	F_F, S
	-	Octadecane	-	P	-	-	-	-
	29	Tetradecane	-	-	F_F, S	F_F, S	F_F, S	F_F, S
	30	Pentadecane	-	P	-	S	-	F_F

	31	Heptadecane	-	P	F_F, S	F_F	S	-
	32	1,1'-oxybis- Octane	-	-	S	F_F	S	-
	47	1-chloro- Octadecane	-	-	F_F	-	-	-
	33	Nonadecane	-	-	F_F, S	F_F	-	-
	34	Hexadecane	-	-	-	F_F, S	F_F	F_F, S
	48	Dodecamethyl- cyclohexasiloxane,	-	-	-	-	-	F_F
Others	49	2,3-dimethyl undecan	-	-	-	-	-	F_F
	59	2-methyl-naphthalene	-	-	S	S	S	-
	60	Naphthalene	-	-	-	S	S	S
	35	2,3- dihydro- benzofuran,	-	P	F_F, S	F_F, S	F_F, S	F_F, S
	36	Methylene chloride	-	P	F_F, S	F_F, S	F_F, S	F_F, S
	-	Morpholine,	-	P	-	-	-	-
	-	1-Octyl trifluoroacetate	-	P	-	-	-	-
	37	2-Ethylhexyl salicylate	-	P	F_F	F_F	F_F, S	F_F, S
	38	Dimethyl ether	-	-	S	F_F	F_F	-
	61	2,4,6-trimethyl-pyridine	-	-	-	-	-	S

1 F_F = Final fermentation (time 24 h); S = Storage time (28 days); P= Compound present in the substrate; - = Flavor not found / compound not detected.

To correlate the volatile compounds with the different fermentation assays at the end of the fermentation (Fig. 3A) and storage periods (Fig. 3B), principal component analysis was performed. At end of fermentation, as shown in Fig. 3A, the PC1 and PC2 explain 42.43 % and 32.97%, respectively, of the total variance. On the positive side of PC2, the assay inoculated with LAB was mainly correlated to (Z)-6,10-dimethyl, 5,9-undecadien-2-one, 2-nonen-1-ol, 1-chloro- octadecane, n-heptadecanol-1, 4-ethyl-1,3-benzenediol, nonanal and isophorone. On the negative side of PC2 and the negative side of PC1, the assays inoculated with LAB and the two *S. cerevisiae* strains were grouped together and mainly correlated with the following volatile compounds: 1-octanol, ethanol,1-(2,4-eimethyl-furan-3-yl)-ethanone, 2-methyl -1-butanol, 3,5-dimethyl-2-furyl methyl ketone; and hexanoic acid. These assays showed the highest number of volatile compounds detected in the present study. On the negative side of PC2 and the positive side of PC1, the assay with LAB and *P. kluyveri* was mostly correlated with 1-pentanol, 1-butanol, 3-methyl- acetate, pentadecane, homosalate, dodecamethyl-cyclohexasiloxane, and 2,3-dimethyldecane.

After 28 days of storage, there were modifications on the profile of volatile compounds of the beverages produced from different microbial assays as observed by the modification in the PCA groups (Fig. 3B). For example, the compounds (Z)- 2 dihydro-5-(2-octenyl)- (3H)-furanone; 2-methyl-naphthalene; Naphthalene; (Z)-3-Decen-1-ol; Cyclodecanol, which were not detected at end of fermentation, were found at 28 day of storage. Condurso, Verzera, Romeo, Ziino, & Conte (2008) reported that volatile compounds are formed due to numerous biochemical changes that occur during the process of fermentation and also during storage of the product. The PC1 and PC2 explain 40.76 % and 36.93%, respectively, of the total variance. On the positive side of PC2 and the negative side of PC1, the assay with a single culture of LAB was correlated

to 1-dodecanol, 1-hexadecanol, 3-methyl- butanoic acid, 1,1'-oxybis-octane, heptadecane, methylene chloride, dimethyl ether and (E)-2-decenal which were different from those predominant at the end of fermentation. The assays of LAB and the two *S. cerevisiae* strains were grouped on the negative side of PC2 and the positive side of PC1. However, the assay with LAB and *S. cerevisiae* CCMA 0731 was more correlated with 1-heptanol, 3-methyl-1-butanol, 1-tetradecanol, pentadecane, (Z)-3-decen-1-ol and cyclodecanol compounds, while the assay with LAB and *S. cerevisiae* CMMA 0732 was more correlated with 2-nonen-1-ol, 1-pentanol, and 2-propyl-1-pentanol compounds. The volatile compounds (Z)-6,10-dimethyl-5,9-undecadien-2-one, 2(3H)-furanone, dihydro-5-(2-octenyl)- (Z)- were found in both assays. On the positive sides of PC1 and PC2, the assay inoculated with LAB and *P. kluyveri* was correlated to 1-(2,4-dimethyl-furan-3-yl)-ethanone, 3-methyl-1-butanol acetate, tetradecane, 2,4,6-trimethyl- pyridine compounds.

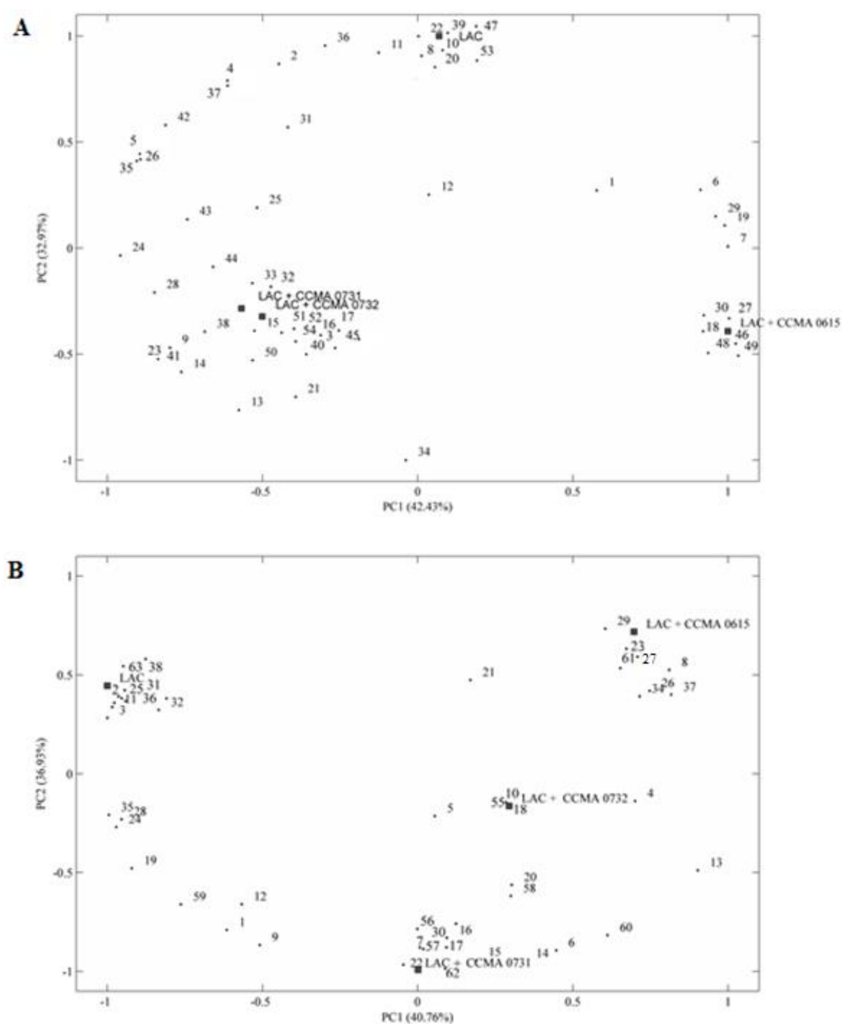

Fig. 3. Principal component analysis loading plot of the following main compounds during the fermentation. Final fermentation time (24 h) (A), 28 days refrigerated storage (B). (1) 2-ethyl-1-hexanol; (2) 1-Dodecanol; (3)2-(dodecyloxy)-ethanol; (4) 2,4-bis (1,1-dimethylethyl)-phenol; (5) 1-Decanol ; (6) 1-Hexanol; (7) 1-Heptanol; (8) 4-ethyl-1,3-Benzenediol; (9) 1-Octanol; (10) 2-Nonen-1-ol; (11) 1-Hexadecanol; (12) 1-Nonanol, (13) Phenylethyl Alcohol; (14) 2-methyl-1-butanol; (15) Ethanol; (16) 3-methyl-1-Butanol; (17) 1-Tetradecanol; (18) 1-Pentanol, (19) 2,4-dimethyl-Benzaldehyde; (20) (Z)- 6,10-dimethyl-5,9-Undecadien-2-one; (21) 6-methyl-5-Hepten-2-one; (22) Isophorone; (23) 1-(2,4-Dimethyl-furan-3-yl)-ethanone; (24) Acetic acid; (25) 3-methyl-butanoic acid; (26) 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester; (27) 3-methyl, 1-butanol acetate; (28) decamethyl-cyclopentasiloxane; (29) Tetradecane; (30) Pentadecane; (31) Heptadecane; (32) 1,1'-oxybis- octane; (33) Nonadecane; (34) Hexadecane; (35) 2,3-dihydro-benzofuran; (36) Methylene chloride; (37) 2-Ethylhexyl salicylate; (38)

Dimethyl ether; (39) n-heptadecanol-1; (40) (Z)-2-Heptenal; (41) 3,5-Dimethyl-2-furyl methyl ketone, (42) 2-hydroxy-benzoic acid; (43) Dodecanoic acid; (44) n-decanoic acid; (45) Formic acid, heptyl ester; (46) Homosalate; (47) 1-chloro-octadecane; (48) Dodecamethyl-cyclohexasiloxane; (49) 2,3-dimethyl-undecane; (50) Hexanoic acid, (51) n-pentadecanol; (52) n-Nonadecanol-1; (53) Nonanal; (54) (Z)- 3-Nonen-1-ol (55) 2-Propyl-1-pentanol; (56) (Z)-3-Decen-1-ol; (57) 1,4-benzenedicarboxaldehyde; (58) (Z)-dihydro-5-(2-octenyl)-2(3H)-furanone; (59) 2-methyl-naphthalene; (60) Naphthalene; (61) 2,4,6-trimethyl-pyridine; (62) Cyclodecanol; (63) (E)-2-decene

3.4 Sensory evaluation of beverages

The consumer acceptance of the different produced maize beverages was evaluated, and the results are shown in Table 3. Flavor, taste, and overall impression did not differ significantly ($p > 0.05$) among the different beverages. Although the profile of volatile compounds profile and organic acids were different among the assay, probably their concentration may be low to be perceived by untrained consumers. A training section with some food ingredients related to the flavor and taste of the detected compounds could be indicated to detect minor differences. Similar results were obtained by Freire et al. (2017) producing cassava-based beverage using co-culture of yeasts and LAB. The appearance and texture of the beverage produced with LAB and *S. cerevisiae* CCMA 0732 produced a lower score ($p < 0.05$) than the others. Although the volatile compound profiles were different among the assays, the untrained panel was not able to differentiate the samples. In general, the consumers neither disliked nor liked (score 5) the beverages. Considering that the beverages had no flavoring additives and there was an absence of the sweet taste normally present in fermented foods, such as yogurt and fermented milks, this is an interesting result, and it indicates that the maize substrate could be used for developing novel beverages. However, the addition of fruit pulps,

flavorings and sweeteners to the beverages could be an alternative to enhance their aroma and consequently consumer acceptance.

Table 3. Acceptance of maize based beverages fermented with *L. paracasei* LBC-81 and three different starter cultures in co-culture with *L. paracasei* LBC-81.

Beverages	Sensory Attributes				
	Appearance	Flavor	Taste	Texture	Overall impression
Control	5,93^{b}	5,50^{a}	4,82^{a}	5,87^{b}	5,25^{a}
L. paracasei	5,55^{ab}	5,48^{a}	4,82^{a}	5,65^{ab}	5,15^{a}
***L. paracasei + Saccharomyces cerevisiae* CCMA 0732**	5,46^{a}	5,52^{a}	4,57^{a}	5,40^{a}	5,07^{a}
***L. paracasei + Saccharomyces cerevisiae* CCMA 0731**	5,80^{ab}	5,23^{a}	4,68^{a}	5,67^{ab}	5,08^{a}
***L. paracasei + Pichia membranifaciens* CCMA 0615**	5,80^{ab}	5,63^{a}	4,97^{a}	5,62^{ab}	5,45^{a}

The scores for the consumer acceptance test are the mean ± SD (n=50). Values with a different letter are significantly different (P< 0.05) according to Tukey test. Acceptability was evaluated using a structured hedonic scale of 9 points, from 1 (dislike very much) to 9 (like very much).

4 Conclusion

The co-incubation of the probiotics cultures of *L. paracasei* LBC-81 with *S. cerevisiae* CCMA 0731 and *L. paracasei* LBC-81 with *S. cerevisiae* CCMA 0732 in the maize substrate maintained a viable probiotic population (≥ 10^{6} CFU / mL) during fermentation and storage under refrigeration for 28 days. Organic acids, such as lactic and acetic acids, were produced and maintained the low pH (around 4.0) of the beverage, which is important for the food safety, taste, and aroma of the beverages. Furthermore, these assays showed the highest number of volatile compounds; however,

it was not noted by the consumers and they neither disliked nor liked the beverages which is a positive result since no flavoring was added to the beverages. To improve the consumers acceptance, the addition of fruit pulps, flavorings and sweeteners could be indicated and evaluated in the next studies.

The combination of probiotic strains of yeast and LAB was employed for the elaboration of non-dairy fermented beverages. The researchers have characterized and selected potential probiotic LAB and yeasts from different environments; however, only LAB have been evaluated for food fermentation. The present study showed the possibility of successfully obtaining a maize-based beverage fermented by both probiotic strains of LAB and yeast. However, further studies on the viability and benefits of these strains in the gut after consumption, as well as improving the beverage acceptability to consumers, should be conducted.

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4 CONCLUSÃO GERAL

Neste trabalho, leveduras isoladas a partir de diferentes fermentações de alimentos brasileiros, foram caracterizadas quanto suas características probióticas. De 116 isolados inicialmente estudados, doze *Saccharomyces cerevisiae* (9 de bebida indígena caxiri e 3 de kefir) e uma *Pichia kluyveri* (fermentação de cacau) apresentaram propriedades potenciais para serem utilizados como probióticos, sendo capazes de resistir as condições adversas impostas, como, pH ácido, sais biliares e temperatura corporal, assim como foram capazes de se aderir a células Caco-2. As leveduras *S. cerevisiae* CCMA 0731, *S. cerevisiae* CCMA 0732 e *P. kluyveri* CCMA 0615, foram selecionadas entre as 12 cepas, por apresentarem alta capacidade de adesão, alta atividade antioxidante e por produzem fitase, estas leveduras foram submetidas a testes de inibição de patógenos alimentares *Escherichia coli* EPEC, *Salmonella* Enteritidis e *Listeria monocytogenes*, o efeito inibitório foi dependente da cepa e do tratamento estudado (ensaios de exclusão e competição). Embora ambos os métodos tenham mostrado efeitos positivos CCMA 0615 foi mais eficiente contra EPEC no teste de exclusão, CCMA 0731 foi mais eficiente no teste de competição com *L. monocytogenes* e tão eficiente quanto a cepa comercial nos testes de exclusão e competição com *S. Enteritidis*. Estas três leveduras foram co-incubadas com culturas probióticas de *Lactobacillus paracasei* LBC-81 em substrato de milho para produção de uma bebida fermentada probiótica não láctea, a população manteve-se viável (≥ 106 UFC/mL) durante a fermentação e armazenamento sob refrigeração por 28 dias nas fermentações com *S. cerevisiae*. Além disso, esses ensaios mostraram o maior número de compostos voláteis; no entanto, isso não foi notado pelos consumidores e eles não gostaram nem gostaram das bebidas, o que é um resultado positivo, uma vez que nenhum sabor foi adicionado. Ácidos orgânicos, como ácidos lático e acético, foram produzidos e mantiveram o pH baixo (em torno de 4,0) da bebida, o que é importante para a segurança alimentar, sabor e aroma das bebidas. Embora o *Lactobacillus* seja o microrganismo probiótico mais utilizado pelas indústrias, as leveduras podem apresentar outras vantagens, como a alta atividade antioxidante, produção de enzimas, como a fitase, assim como a prevenção e tratamento de infecções alimentares por bactérias patogênicas.