GUSTAVO FELIPE CORREIA SALES

DIVERSIDADE E CARACTERIZAÇÃO PROBIÓTICA DE LEVEDURAS ENCONTRADAS NO TRATO GASTROINTESTINAL DE BOVINOS

LAVRAS - MG

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GUSTAVO FELIPE CORREIA SALES

DIVERSIDADE E CARACTERIZAÇÃO PROBIÓTICA DE LEVEDURAS ENCONTRADAS NO TRATO GASTROINTESTINAL DE BOVINOS

DIVERSITY AND PROBIOTIC CHARACTERIZATION OF YEASTS FOUND IN THE GASTROINTESTINAL TRACT OF CATTLE

Defesa de tese apresentada à Universidade Federal de Lavras, como parte das exigências do programa de pós-graduação em Microbiologia Agrícola, para a obtenção do título de Doutor.

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2022

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RESUMO

O uso de leveduras como suplemento alimentar para bovinos pode auxiliar no desenvolvimento e melhorar o desempenho do hospedeiro. Porém, para que os resultados benéficos sejam consistentes é necessário selecionar cepas com características probióticas desejáveis. O presente estudo teve como objetivo isolar e identificar leveduras presentes no intestino de bovinos, e avaliar, junto com cepas previamente isoladas do rúmen (estudo preliminar), o potencial probiótico para bovinos. O total de 193 cepas foram avaliadas, sendo 139 cepas (pertencentes a 19 espécies) isoladas de amostras de fezes de onze diferentes animais, e 54 cepas previamente isoladas do líquido ruminal. A população de leveduras nas fezes variou de 3.51 a 4.99 log UFC/g, sendo a espécie *Candida pararugosa* a mais abundante e mais frequente (isolada das fezes de seis amostras no total de onze analisadas). Foram selecionadas cepas que apresentaram resultado negativo para testes de segurança (atividade hemolítica, DNAse e gelatinase) e porcentagens superiores a 35 e 70% de hidrofobicidade e autoagregação, respectivamente. Foram selecionadas cepas com porcentagem de coagregação superior a 77.7 e 74.7%, com cepas patogênicas de *Escherichia coli* e *Clostridium perfringens*, respectivamente. Foram selecionadas cepas que apresentaram porcentagem de crescimento relativo a 39ºC superior a 61% e taxa de viabilidade maior que 96.7% para o teste de sobrevivência nas condições gastrointestinais de bovinos. Foram selecionadas as sete cepas que apresentaram os melhores resultados para os testes de seleção, sendo estas pertencentes as espécies, *Candida pararugosa* (L60, CCMA 928 e CCMA 930) e *Pichia kudriavzevii* (isolados L97, L100, CCMA904, CCMA 907). As cepas selecionadas são produtoras de exopolissacarídeos. De acordo com os resultados dos atributos avaliados, as sete cepas selecionadas foram consideradas com potencial probiótico para desempenhar seus benefícios em bovinos.

Palavras-chave: Diversidade; Adesão celular; Inibição de patógenos; Estímulo do sistema imune.

ABSTRACT

The use of yeasts as a feed supplement for cattle can promote development and improve host performance. However, for the positive results to be consistent, strains with desirable probiotic properties must be selected. The objective of the present study was to isolate and identify yeasts present in the intestine of cattle and, together with strains previously isolated from the rumen (preliminary study), to evaluate their probiotic potential for cattle. A total of 193 strains were studied, including 139 strains (belonging to 19 species) isolated from fecal samples from 11 different animals and 54 strains previously isolated from rumen fluid. The yeast population in the feces ranged from 3.51 to 4.99 log CFU/g, with *Candida paraugosa* being the most abundant and frequent (isolated from the feces of six of the eleven samples analyzed). Strains were selected that were negative results in the safety tests (hemolytic activity, DNAse, and gelatinase) and had percentages greater than 35 and 70% for hydrophobicity and autoaggregation, respectively. None of the strains evaluated produced antipathogenic metabolites, but strains with a percentage of coaggregation greater than 77.7 and 74.7% were selected, which were pathogenic strains of *Escherichia coli* and *Clostridium perfringens*, respectively. The strains that had a percentage of growth relative to 39ºC greater than 61% and viability greater than 96.7% were selected for survival testing under the gastrointestinal conditions of cattle. After the tests, the seven best strains were selected, belonging to the species *Candida paraugosa* (L60, CCMA 928, and CCMA 930) and *Pichia kudriavzevii* (isolated L97, L100, CCMA904, CCMA 907). The selected strains did not show the ability to produce cellulolytic, amylolytic, or proteolytic enzymes, but are exopolysaccharide producers. Based on the results of the evaluated properties, the seven selected strains were classified as potential probiotics for cattle.

Keywords: diversity; cell adhesion; inhibition of pathogens; stimulation of the immune system.

SUMÁRIO

PRIMEIRA PARTE

INTRODUÇÃO

Os sistemas agropecuários estão em constante evolução, exigindo cada vez mais melhoria no desempenho animal. A crescente demanda por alimentos de origem animal destacam a necessidade de alternativas para o gerenciamento desses sistemas no presente e no futuro (Alexandratos e Bruinsma, 2012). O alto desempenho animal está fortemente associado ao equilíbrio da microbiota do trato gastrointestinal, que possui grande participação no seu ganho energético (Bergman, 1990; Dehority, 2003).

A maioria das práticas atualmente utilizadas para modelar a microbiota e modificar a fermentação ruminal envolvem o uso de antibióticos. Há uma estimativa de um aumento de 11.5% no uso de antibióticos na produção de produtos de origem animal entre os anos de 2017 (93.309 toneladas) e 2030 (104.079 toneladas) (Tiseo et al, 2020). Porém, há hoje um receio quanto o resíduo gerado por esses químicos, tanto nos animais quanto no meio ambiente. Esses resíduos podem gerar microrganismos resistentes a antibióticos, com impactos significativos na saúde pública (Van Boeckel et al., 2015). Uma das alternativas para melhoria do rendimento dos animais, apontada como segura e natural é o uso de probióticos, que são microrganismos vivos com capacidade de conferir benefícios para a saúde do hospedeiro (Guillermo et al., 2015, Imperial e Ibama, 2016).

Existem diferentes trabalhos publicados mostrando que a utilização de leveduras como suplemento na alimentação pode gerar benefícios no desenvolvimento e na produtividade de animais de criação (Shurson, 2018). Em relação aos ruminantes, os estudos demostram diferentes benefícios da suplementação de leveduras na dieta, como a redução da concentração de lactato no rúmen, equilíbrio do pH ruminal, estímulo do crescimento de microrganismos celulolíticos e fibrolíticos e aumento da ingestão e digestão de fibras, remoção do oxigênio, aumento da produção de leite e redução da emissão de metano (Chaucheyras-Durand et al., 2008, Sartori et al., 2017; Shurson, 2018).

Apesar dos resultados positivos citados anteriormente, pouco se sabe sobre a diversidade de leveduras encontradas no trato gastrointestinal destes animais e são poucos os trabalhos com o objetivo de selecionar leveduras com potencial probiótico para bovinos. As principais cepas utilizadas como suplemento alimentar para ruminantes pertencem a espécie

Saccharomyces cerevisiae, uma espécie de levedura amplamente utilizada na indústria alimentícia, mas que não foi selecionada especificamente para utilização com suplemento na alimentação de animais ruminantes e não são descritas como autóctone do trato gastrointestinal de animais e humanos (Shurson, 2018; Garcia-Mazcorro et al., 2019; Ghazanfar, 2021).

O conhecimento da diversidade e a caracterização das leveduras é o primeiro passo para uma seleção de probióticos, pois melhora o entendimento do comportamento e modo de ação no TGI dos animais (Chaucheyras-Durand et al., 2008). Neste contexto, é de fundamental importância estudos que descrevam a diversidade de leveduras presentes nas diferentes partes do trato gastrointestinal de bovinos e caracterização e seleção de leveduras com potencial probiótico e biotecnológico.

REFERENCIAL TEÓRICO

Microbiota do trato gastrointestinal de animais ruminantes

Os bovinos são animais herbívoros ruminantes que não têm a capacidade de degradar a celulose e outros carboidratos estruturais das plantas. Esses animais desenvolveram relação de simbiose com uma grande comunidade microbiana e por meio dessas relações conseguem obter energia a partir de determinados compostos de origem vegetal. O alimento consumido pelos bovinos fornece nutrientes aos microrganismos, além de contribuir para a manutenção de condições físicas e químicas para a fermentação microbiana ideal. Em troca, as comunidades microbianas presentes no trato gastrointestinal (TGI) estão envolvidas na digestão e fermentação de polímeros vegetais, na síntese de vitaminas, na bioconversão de compostos tóxicos em resíduos não tóxicos, na estimulação do sistema imunológico, manutenção do peristaltismo intestinal e integridade da mucosa intestinal, além de desempenhar um papel importante contra a colonização por patógenos (Bergman, 1990; Dehority, 2003).

O TGI dos ruminantes inclui um estômago formado por quatro compartimentos, que consiste no rúmen, retículo, omaso e o abomaso. A fermentação microbiana das fibras e dos alimentos sólidos ocorre no rúmen, enquanto os líquidos são transferidos para o retículo, que serve também para o aprisionamento de grandes partículas de alimento, posteriormente regurgitado para ótima digestão. No omaso, os líquidos são filtrados e vários nutrientes são absorvidos e, finalmente, no abomaso ocorre a digestão enzimática do alimento (Hofmann,

1989). Após o estômago compartimentado o TGI pode ser dividido em duas regiões, intestino delgado (duodeno, jejuno e íleo), intestino grosso (ceco, colón e reto).

Existe uma comunidade densa e complexa de microrganismos em todo o TGI dos ruminantes, sendo que a maior diversidade microbiana é observada no rúmen, onde é realizada a fermentação microbiana. A microbiota ruminal é composta por microrganismos pertencentes aos três domínios; Bacteria, Archaea (incluindo metanogênicas) e Eukarya (protozoários e fungos) (Nagaraja, 2016). Muitos desses organismos que colonizam e crescem no trato digestivo são considerados nativos e, portanto, são chamados de microbiota autóctone. Além disso, a microbiota ruminal inclui também microrganismos transitórios, derivados em grande parte de alimentos e água ingeridos e, em pequena extensão, do ar ingerido ou de outro habitat do hospedeiro (pele, trato respiratório ou trato reprodutivo). Esses microrganismos transitórios não se estabelecem (não há colonização e crescimento), e são denominados alóctones (Stewart et al., 1988).

No rúmen, as leveduras são encontradas nas concentrações de 10^2 a 10^3 por grama de conteúdo ruminal e são geralmente consideradas como população transitória e não contribuem diretamente para a fermentação ruminal (Nagaraja, 2016). No entanto, o número de leveduras aumenta no rúmen de animais com acúmulo de ácido lático, provavelmente devido à disponibilidade de açúcares altamente fermentáveis (Nagaraja, 2016). A temperatura corporal média dos bovinos é em torno de 39 ºC, mais alta que a temperatura ótima para crescimento de leveduras mesófilas, que é entre 20-30ºC. Lund (1974) observou maior crescimento de leveduras no líquido ruminal incubadas a 25ºC quando comparado com o crescimento a 39ºC.

Fernandes et al. (2019) isolaram, identificaram e caracterizaram leveduras presentes no líquido ruminal e selecionaram cepas que apresentaram potencial como probióticos. A população de levedura variou de 3,84 a 6,76 $\log_{10}$UFC ml^{-1}. As principais espécies de leveduras identificadas foram *Pichia kudriavzevii, Candida rugosa, Ca. pararugosa, Ca. Etanolica* e *Magnusiomyces capitatus.* Lund (1974) também isolou leveduras do líquido ruminal de bovinos com diferentes dietas e identificaram cepas pertencentes as espécies *Trichosporon cutaneurn, T. capitaturn, Pichia kudriavzevii, P. membranaefaciens, Candida ingens, Toruiopsis pintolopesii, Kluyveromyces bulgaricus, Saccharomycopsis lipolytica* e *Hansenula fabianii*. O autor observou que animais que estavam consumindo dietas a base de silagem, apresentaram maior diversidade de leveduras, em comparação com aqueles consumindo gramíneas frescas.

Em ambos os estudos, a espécie *Pichia kudriavzevii* foi a mais abundante entre as leveduras isoladas.

Abrão et al. (2014) isolaram e caracterizaram de fungos do fluido ruminal de bovinos de corte de diferentes idades, criados em pastagens tropicais lignificadas. Os autores isolaram e agruparam as leveduras através de sua capacidade fermentativa. As espécies identificadas foram *Torulaspora globosa, Wickerhamomyces anomalus, Pichia kudriavzevii* e *Cryptococcus laurentii*. Além de identificar os microrganismos, os autores concluíram que os isolados de levedura de vaca (4 anos de idade) mostraram capacidade de catabolizar uma maior diversidade de fontes de carbono e nitrogênio, quando comparado com bezerros (6 a 8 meses de idade) e novilhos (24 a 40 meses de idade).

A fermentação ruminal equilibrada é essencial na manutenção da saúde e produtividade dos ruminantes, uma vez que vários parâmetros fisiológicos dos animais de produção estão altamente correlacionados com a abundância de vários membros da microbiota ruminal (Jami et al. 2014). Alguns fatores, como as práticas de alimentação, composição das dietas e manejo podem influenciar o balanço microbiano no TGI e, consequentemente, afetar a eficiência alimentar, o bem-estar digestivo e a saúde dos animais (Chaucheyras-Durand e Durand, 2010). Mudanças de dietas baseadas em forragem para dietas com alto valor energético pode induzir modificações na microbiota ruminal, que podem estar associadas ao aumento do risco de acidose ruminal (Nagaraja e Titgemeyer, 2007). O desmame também representa um período crítico durante o qual a microbiota intestinal ainda imatura tem que enfrentar uma mudança abrupta na dieta, o que leva a um aumento na suscetibilidade dos animais jovens à colonização de patógenos (Zoetendal et al., 2008).

São poucos os trabalhos que isolaram e caracterizaram os microrganismos presentes no TGI pós rúmen. Kim e Wells (2016) realizam uma meta-análise de sequências do gene rRNA16S de origem fecal bovina que estão disponíveis publicamente no banco de dados do RDP. Os autores descrevem a diversidade de procariotos identificados em amostras de fezes bovinas e observaram que apenas 6,5% das unidades taxonômicas operacionais avaliadas eram compartilhadas entre o rúmen e as fezes. Van Uden e Carmo-Sousa (1957) avaliaram a diversidade de levedura do ceco de 252 bovinos adultos e foram isoladas 131 cepas pertencentes as espécies, *Saccharomyces cerevisiae, S. chevalieri, S. drosophilarum, S. fragilis, Pichia bovis, P. membranaefaciens, P. fermentans, Cryptococcus difluens, Torulopsis glabrata,*

Torulopsis sp., Candida tropicalis, Ca. parapsilosis, Ca. krusei, Ca. macedoniensis, Ca. utilis, Ca. bovina e *Trichosporon cutaneum*. Lopes et al. (2019) avaliaram dinâmica da microbiota em todo o trato gastrointestinal de novilhos Nelore e a análise de beta diversidade mostrou que as comunidades fúngicas do rúmen diferiram entre as frações líquida e sólida. Além disso, as comunidades fúngicas das frações líquida e sólida do rúmen também foram distintas das encontradas nos segmentos distais do TGI (intestino delgado, ceco e amostras fecais). No entanto, não foram observadas diferenças entre as comunidades fúngicas no intestino delgado, ceco e amostras fecais.

Angchuan et al. (2021) realizaram uma ampla caracterização de leveduras presentes no intestino de bovinos de corte na Tailândia. Os autores estudaram a diversidade de leveduras utilizando métodos dependentes de cultivo. Foram isoladas leveduras do líquido e no epitélio de diferentes regiões do intestino delgado (duodeno, jejuno, íleo). Os autores isolaram 701 leveduras do líquido coletado de 18 amostras do duodeno (279), 18 amostras do jejuno (200) e 18 amostras do íleo (222). Em relação ao epitélio intestinal foram isoladas 425 leveduras de 17 amostras do duodeno (162), 14 amostras do jejuno (139) e 15 amostras do íleo (124). A espécie *Candida parapsilosis* apresentou a maior frequência de ocorrência nas amostras do líquido do duodenal (83,3%), jejunal (77,8%) e íleo (61,1%) e também foi a espécie com maior frequência de ocorrência no epitélio do duodeno (88,2%), jejuno (87,5%) e íleo (87,2%). O coeficiente de similaridade de Jaccard foi utilizado para avaliar a similaridade das comunidades de leveduras associadas ao líquido e ao epitélio do intestino delgado. Com base no coeficiente de Jaccard foi realizada uma análise das coordenadas principais (PCoA) e os autores concluíram que não houve diferença entre a comunidade de leveduras pertencentes ao líquido e ao epitélio das amostras coletadas no duodeno, jejuno e íleo.

A diversidade de procariotos presente no TGI de ruminantes, principalmente a diversidade de bactérias encontradas no rúmen, é bem descrita na literatura. Há vários trabalhos mostrando a diversidade de bactérias e a influência de diferentes fatores sobre estes microrganismos. Porém, a diversidade e a funcionalidade de leveduras ainda permanecem pouco exploradas. Apesar de estarem em baixa população no TGI, as leveduras são microrganismos autóctone deste ecossistema. Conhecer sua diversidade e as alterações que podem ocorrer com diferentes populações, em decorrências de fatores intrínsecos e extrínsecos do hospedeiro, é importante para entender a funcionalidade das leveduras e como sua modulação pode melhorar o desempenho dos animais. Além disto, o cultivo de leveduras

isoladas de diferentes regiões do TGI dos ruminantes, permite analisar o potencial biotecnológico e probiótico destes microrganismos.

Probióticos

O termo probiótico (palavra grega = "para vida") foi inicialmente definido como compostos ou extratos capazes de estimular o crescimento microbiano. Posteriormente, o termo foi descrito como "as substâncias secretadas por um microrganismo que estimula o crescimento de outro", no entanto, o termo foi contrastado com os antibióticos e devido a este fato, esta definição deixou de ser utilizada (Syngai et al., 2016). Em 2001, um grupo de especialistas pertencentes à Organização das Nações Unidas para Agricultura e Alimentação (FAO) e à Organização Mundial da Saúde (OMS), definiram os probióticos como "microrganismos vivos que, quando administrados em quantidades adequadas, conferem um benefício à saúde do hospedeiro" (Hill et al., 2014). O termo prebiótico é mais recente e Bindels et al. (2015) propuseram a definição, "um composto não digerível que, através de sua metabolização por microrganismos no intestino, modula a composição e/ou atividade da microbiota intestinal, conferindo um efeito fisiológico benéfico ao hospedeiro".

A principal aplicação dos probióticos ainda é associar a alimentos lácteos fermentados para o consumo humano. Recentemente, outras aplicações, como suplementos nutricionais destinados a animais, ganharam mais atenção, favorecendo o mercado mundial de probióticos, que ultrapassou 30 bilhões de dólares em 2015, e a previsão era de um valor por volta de 52 bilhões em 2020. O mercado de probiótico destinado exclusivamente a suplementação animal foi avaliado em 3,25 bilhões de dólares em 2016 e deverá crescer 7,7% de 2017 a 2022, chegando a US $ 5,07 bilhões até 2022 (Market Research Report 2016).

Alguns probióticos podem produzir macro (nitrogênio, fosforo e enxofre) e micronutrientes (ferro, zinco, manganês e selênio) que são fatores de crescimento de microrganismos benéficos da microbiota intestinal. Além de interagir e estimular outros microrganismos, os probióticos também interagem com o hospedeiro, influenciando a resposta imune ou produzindo componentes capazes de afetar positivamente o desenvolvimento da mucosa ou o metabolismo das células intestinais do hospedeiro (Delcenserie et al., 2008). A liberação de produtos antimicrobianos, como bactericidas, que inibem o crescimento de bactérias patogênicas, também pode ser um dos mecanismos de ação das cepas probióticas. Outro modo de ação é a competição por nutrientes e espaço, onde a maior afinidade por

nutrientes ou maior adesão ao substrato, característicos das cepas probióticas, podem excluir microrganismos patogênicos (Chaucheyras-Durand e Durand, 2010). Os probióticos também podem auxiliar na desintoxicação de certos compostos inibitórios, como aminas e micotoxinas (Chaucheyras-Durand e Durand, 2010; Shurson, 2018).

Probióticos em ruminantes

O Ministério da Agricultura, Pecuária e Abastecimento do Brasil (MAPA), por meio da instrução normativa 13/2004, define probiótico destinado a animais como "aditivo para produtos destinados à alimentação animal". O aditivo deve: a) ser indispensável à adequada tecnologia de fabricação do produto; b) influir positivamente nas características do produto destinado à alimentação animal, de produtividade dos animais ou dos produtos de origem animal; c) ser utilizado na quantidade estritamente necessária à obtenção do efeito desejado, respeitada a concentração máxima que vier a ser fixada; d) ser previamente autorizado e registrado pela autoridade competente do MAPA. Além disso os aditivos deverão obedecer ao padrão de identidade e pureza, segurança e especificações, fixados pelo Chemical Abstracts Service - CAS, Food Chemicals Codex - FCC (Ministério da Agricultura, Brasil, 2004).

Sabe-se que os efeitos probióticos são específicos de cada cepa, portanto, é importante que as cepas destinadas a aplicação animal sejam cuidadosamente selecionadas. Existem requisitos para que as cepas probióticas possam se adaptar ao ambiente intestinal de uma espécie animal, por exemplo, a capacidade de sobrevivência em todo o intestino, a tolerância aos sais biliares e afinidade com a mucosa e glicoproteínas intestinais (Chauceyras-Durand et al., 2008). É importante que os microrganismos introduzidos não perturbem a população nativa, que já é adaptada ao ambiente do trato gastrointestinal. Os probióticos têm que possuir a capacidade de melhorar a saúde intestinal, sendo capaz, por exemplo, de estimular o desenvolvimento da microbiota saudável, aumentar a capacidade digestiva, prevenir a colonização do intestino por patógenos (Newbold, 1996).

Os modernos sistemas de produção intensiva de carne bovina e laticínios exigem o rápido crescimento muscular e a alta produção de leite, tornando a densidade energética das dietas maior, para suprir as necessidades nutricionais. Nesse contexto, é maior o risco de distúrbios associados ao desequilíbrio do ecossistema microbiano do trato gastrointestinal (TGI), causando redução no consumo da dieta, problemas de saúde e baixa produtividade. O uso de antibióticos é umas das práticas utilizadas com a finalidade de modular a microbiota do

TGI, no entanto, seu uso na alimentação animal aumentou gradualmente as preocupações com os resíduos de antibióticos em produtos de origem animal e o surgimento de microrganismos resistentes aos medicamentos (Papatsiros et al., 2013). Um estudo realizado em diferentes países europeus mostrou uma forte correlação entre os níveis do uso de oito classes de antimicrobianos e a prevalência de *Escherichia coli* comensal resistente a antimicrobianos em porcos, aves e bovinos (Chantziaras et al., 2014).

O uso de probióticos é uma excelente alternativa ao uso de antibióticos e, nos últimos anos, houve aumento do uso de probióticos na produção pecuária, especialmente na União Europeia, onde o uso de antibióticos nesse campo foi completamente proibido (European Parliament and Council, 2003; Jouany e Morgavi, 2007; Papatsiros et al., 2013). No entanto, alguns países, por exemplo, China e EUA, ainda empregam antibióticos na produção animal, e há uma estimativa que para o Brasil, Rússia, Índia, China e África do Sul, o aumento do consumo de antimicrobianos seja de 99% até 2030. (Van Boeckel et al., 2015).

Para convencer pecuaristas, consumidores e legisladores a reconhecer os benefícios dos probióticos numa escala muito mais ampla, é importante o esclarecimento dos mecanismos de ação e propriedades benéficas apresentadas por esses microrganismos, garantindo que os efeitos benéficos sejam consistentes. Mais estudos sobre a estrutura e atividade da microbiota intestinal, interações funcionais entre os microrganismos do intestino, e as relações entre microbiota e as células do hospedeiro são necessários para estabelecer métodos de seleção e determinar os aspectos fundamentais da ação e resultados do uso de probiótico.

Uso de leveduras como probióticos para bovinos

Na literatura há vários registros dos benefícios da utilização de leveduras como suplemento na dieta de ruminantes. Estes benefícios podem ser resultado da ação das leveduras na fermentação e no ambiente ruminal, e no intestino do animal hospedeiro.

Apesar das cepas de levedura serem consideradas microrganismos transitórios no rúmen e não possam colonizar adequadamente o rúmen por um longo período, Kung et al. (1997) observaram que *S. cerevisiae* era metabolicamente ativa no fluido ruminal por até 48 horas. As leveduras têm o potencial de desempenhar duplo papel no rúmen, probiótico e prebiótico. A ação probiótica da levedura no rúmen pode ser explicada através da modulação da composição e atividades do ecossistema microbiano ruminal, levando à diminuição do

conteúdo de ácido láctico, ao aumento e manutenção do pH ruminal, à melhora na digestibilidade dos nutrientes, à otimização dos perfis de ácidos graxos voláteis e a um decréscimo na produção de amônia ruminal (Wang et al., 2016). Outro efeito positivo da levedura no rúmen é alterar a fermentação ruminal para reduzir a produção de metano, diminuindo as perdas de energia durante a fermentação (Ruiz et al., 2016).

Leveduras podem realizar a redução do oxigênio no rúmen, criando um ambiente mais anaeróbico exigido pelos microrganismos ruminais. Nesse contexto, a levedura em si funciona não apenas como um probiótico, mas também ajuda outros membros da comunidade ruminal a crescer e, portanto, atua como um tipo de prebiótico. Cepas de *Saccharomyces cerevisiae* mutantes (NCYC 694 e NCYC 1088), incapazes de remover oxigênio do fluido ruminal, não estimularam aumento do número de bactérias em simuladores da fermentação ruminal, porém as linhagens parentais (NCYC 240 e NCYC 1026), capazes de eliminar oxigênio, reduziram a concentração de O_2 em 85,3 e 43,1% em relação ao controle, respectivamente, e estimularam a atividade bacteriana (Newbold et al., 1996).

A presença de oligossacarídeos, aminoácidos, vitaminas e ácidos orgânicos, contidos nas células de levedura, também provocam efeito prebiótico, estimulando o crescimento da microbiota ruminal, porém são necessários estudos que possam avaliar se a quantidade dessas substâncias produzidas pelas leveduras seja capaz de alterar a fermentação ruminal (Beauchemin et al., 2006).

Alguns mecanismos foram descritos para explicar o efeito positivo da suplementação com leveduras sobre o pH ruminal. Um mecanismo é a capacidade das leveduras de competir pelo mesmo substrato com bactérias formadoras de lactato (ex.: *Streptococcus bovis*) no rúmen (Chaucheyras et al., 1996). Outro mecanismo é a capacidade de leveduras de estimular o crescimento de populações de bactérias consumidoras de lactato, como *Megasphaera elsdenii* (Meissner et al., 2010).

Células viáveis de levedura podem sobreviver à degradação ruminal e à digestão ácida no abomaso e chegar ao intestino delgado (Chaucheyras-Durand et al., 1998). É importante que a levedura percorra todo o TGI e cheguem viáveis no intestino do hospedeiro, porque algumas ações do probióticos podem ser realizados no intestino, como a inibição da infecção por microrganismos patogênicos e o estímulo do sistema imune do hospedeiro. No intestino,

glucanas e mananas presentes na parede celular das leveduras podem exercer um efeito probiótico estimulando a imunidade epitelial inespecífica dos ruminantes (Moran, 2004).

Os patógenos geralmente aderem ao epitélio para estabelecer infecções que podem ser letais, e os probióticos eucarióticos podem impedir que os patógenos se liguem às células intestinais por efeito antagonista direto ou secretando metabólitos. Leveduras podem competir com microrganismos patogênicos por substrato e receptores de mucosa no intestino e, assim, evitar que os patógenos colonizem no intestino do hospedeiro. Leveduras contêm manoproteínas em sua camada externa de sua parede celular e, portanto, podem induzir um efeito protetor contra esses patógenos. Os patógenos se ligam as manoproteínas presentes na superfície da levedura, em vez de se prenderem às células epiteliais intestinais e, em seguida, são eliminados do trato digestivo do hospedeiro após serem aglutinados pelas leveduras (Nayak, 2011).

Os estímulos dos mecanismos imunes humorais podem ser conduzidos pela colonização microbiana no TGI, esses eventos podem promover a circulação das células secretoras de imunoglobulinas (IgA e IgM). O outro fator importante que pode ser afetado pela colonização microbiana no intestino de diferentes animais, especialmente os ruminantes, é o equilíbrio dos diferentes subconjuntos linfócitos T. Quando o hospedeiro é exposto ao antígeno (microrganismo probiótico), as células imunes respondem liberando citocinas do hospedeiro e direcionam as respostas imunes subsequentes (Rigobelo e Ávila, 2012). As leveduras são uma fonte de substâncias biologicamente ativas que podem estimular vários sistemas biológicos, incluindo o sistema imunológico do hospedeiro. Os componentes da parede celular da levedura são predominantemente compostos de polímeros complexos de b-glucanos, a-mananos, manoproteínas e um componente amino da quitina. Essas macromoléculas podem estimular o sistema imunológico do hospedeiro, especialmente a resposta inflamatória e o sistema reticulo endotelial (Nayak, 2011). Apesar destas macromoléculas estarem presentes na maioria das leveduras, a resposta do hospedeiro ocorre de forma específica ao nível de cepas (Ashrafe Shah, 2014). As cepas microbianas probióticos interagem com os tecidos epiteliais do hospedeiro e recrutam as células imunológicas para o local da infecção e induzem marcadores imunológicos (citocinas) específicos, fazendo com que as células ligadas a resposta imune estejam em constante proliferação e permitindo que o sistema imune esteja equilibrado (homeostasia) e preparado para uma rápida resposta (Ashrafe Shah, 2014).

As cepas de levedura mais comumente usadas como suplemento na nutrição de ruminantes pertencem a espécie *S. cerevisiae* (Garcia-Mazcorro et al., 2019). As leveduras estão presentes em baixa concentração no fluido ruminal e nos trabalhos de Lund (1974) e Fernandes et al. (2019) nenhuma das espécies isoladas é de *S. cerevisiae*. Há estudos focados nos efeitos da suplementação com *S. cerevisiae*, mas pouco se sabe sobre o efeito de outras espécies de levedura na fermentação ruminal. Wang et al. (2016) compararam os efeitos de diferentes linhagens de leveduras (*S. cerevisiae, Candida tropicalis* e *Ca. utilis*) na digestão *in vitro* de palha de milho e palha de arroz. Os resultados indicaram que *Ca. tropicalis* foi a cepa com maior capacidade de melhorar a fermentação, como indicado pela redução da matéria seca e fibra em detergente neutro, juntamente com a menor produção de metano.

Foram encontrados dois estudos que analisaram o efeito da suplementação de leveduras vivas sobre a expressão gênica (Bach et al., 2018a) e sobre a microbiota (Bach et al., 2018b) do ambiente ruminal e do cólon de vacas (Holstein) em transição do período seco para a lactação. Bach et al. (2018a) concluíram que a suplementação de leveduras vivas promoveu o aumento da expressão de genes (fator de necrose tumoral α, interleucina 10, antígeno nuclear de célula proliferante e antígeno KI-67) que regulam a inflamação e a barreira epitelial no rúmen. No cólon, a suplementação com leveduras aumentou a expressão do gene β-Defensinas, que codifica para um peptídeo antimicrobiano, que pode melhorar a resposta imune. Em relação a microbiota, Bach et al. (2018b) observaram que a transição para o período de lactação influenciou mais a diversidade de microrganismos do que o efeito da suplementação de leveduras. Mas vacas que consumiram leveduras, apresentaram o dobro da abundância relativa da família *Eggerthellaceae* no cólon, e que estes microrganismos foram positivamente correlacionados com a eficiência alimentar.

Dias et al. (2018) observaram que a suplementação de leveduras para vacas holandesas (em terço médio da lactação) em condições de estresse por calor, melhorou a eficiência alimentar, reduzindo o consumo e mantendo o nível de produção de leite (30.5 kg/d). Os autores concluíram que esses resultados podem ser associados ao aumento da concentração plasmática de niacina que está associada a maior perda de calor corporal.

Apesar de trabalhos demostrarem efeitos benéficos da utilização de leveduras como aditivos para aumento do desempenho de bovinos, foi encontrado apenas dois artigos em que os autores realizaram testes com a finalidade de selecionar leveduras com potencial probiótico

para ruminantes, conduzido por Fernandes et al. (2019) e Suntara et al. (2021). No primeiro experimento, os autores realizaram uma seleção de leveduras isoladas do líquido ruminal coletado em 4 rebanhos (localizados nos estados do Paraná e Minas Gerais, Brasil) de gado leiteiro (Holstein Jersey) e de corte (Nelore), com objetivo de avaliar o crescimento nesse ambiente e a digestibilidade *in vitro* da fibra em detergente neutro (FDN-D), pH e acúmulo de ácido orgânicos o rúmen. As cepas CCMA 933 (*Candida rugosa*) e CCMA 970 (*Ca. pararugosa*) e CCMA 967 (*Ca. etanolica*), apresentaram propriedades de potenciais probióticos para bovinos como, alta taxa de sobrevivência no líquido ruminal e estímulo da produção de ácidos orgânicos no rúmen. No experimento realizado por Suntara et al. (2021), os autores isolaram leveduras do líquido ruminal de novilhas Holstein-Friesian, com o objetivo de selecionar e identificar essas leveduras e medir a eficiência da produção de biomassa e da atividade celulolítica. Foram isoladas 3 cepas identificadas como, *Pichia kudriavzevii* (H-KKU20), *Candida tropicalis* (I-KKU20) e *Galactomyce spp* (C-KKU20), devido sua capacidade de alta produção de biomassa. A cepa pertencente a espécie *P. kudriavzevii* (H-KKU20) apresentou resultados superiores às outras leveduras em termos de produção de biomassa, atividade da enzima celulase e número de células.

Pesquisas futuras precisam abordar o comportamento das células de levedura no ambiente digestivo. A identificação e características metabólicas e fisiológicas específicas exibidas pelas cepas de levedura permitiria um melhor entendimento do seu modo de ação no rúmen e de suas interações no intestino do animal. São necessários também, estudos que tenham como objetivo selecionar probióticos para melhorar a eficiência dos seus benefícios na nutrição e desenvolvimento especificamente para bovinos.

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SEGUNDA PARTE

DIVERSITY AND PROBIOTIC CHARACTERIZATION OF YEASTS FOUND IN THE GASTROINTESTINAL TRACT OF CATTLE

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ABSTRACT

The use of yeasts as a feed supplement for cattle can promote development and improve host performance. However, for the positive results to be consistent, strains with desirable probiotic properties must be selected. The objective of the present study was to isolate and identify yeasts present in the intestine of cattle and, together with strains previously isolated from the rumen (preliminary study), to evaluate their probiotic potential for cattle. A total of 193 strains were studied, including 139 strains (belonging to 19 species) isolated from fecal samples from 11 different animals and 54 strains previously isolated from rumen fluid. The yeast population in the feces ranged from 3.51 to 4.99 log CFU/g, with *Candida paraugosa* being the most abundant and frequent (isolated from the feces of six of the eleven samples analyzed). Strains were selected that were negative results in the safety tests (hemolytic activity, DNAse, and gelatinase) and had percentages greater than 35 and 70% for hydrophobicity and autoaggregation, respectively. None of the strains evaluated produced antipathogenic metabolites, but strains with a percentage of coaggregation greater than 77.7 and 74.7% were selected, which were pathogenic strains of *Escherichia coli* and *Clostridium perfringens*, respectively. The strains that had a percentage of growth relative to 39ºC greater than 61% and viability greater than 96.7% were selected for survival testing under the gastrointestinal conditions of cattle. After the tests, the seven best strains were selected, belonging to the species *Candida paraugosa* (L60, CCMA 928, and CCMA 930) and *Pichia kudriavzevii* (isolated L97, L100, CCMA904, CCMA 907). The selected strains did not show the ability to produce cellulolytic, amylolytic, or proteolytic enzymes, but are exopolysaccharide producers. Based on the results of the evaluated properties, the seven selected strains were classified as potential probiotics for cattle.

Keywords: diversity; cell adhesion; inhibition of pathogens; stimulation of the immune system.

1 - INTRODUCTION

Studies have shown the beneficial effects of using yeast in ruminant diets (Poppy et al., 2012; Sartori et al., 2017; Shurson, 2018). Yeasts can influence rumen fermentation by, for example, balancing the concentration of lactic acid and oxygen in the rumen, stimulating the growth of fibrolytic and cellulolytic microorganisms, and improving fiber digestion (Desnoyers et al, 2009; Sartori et al, 2017; Ghazanfar et al, 2021). These microorganisms may also have beneficial effects in the gut by competing with and inhibiting colonization of pathogens or stimulating the host immune system through their interaction with cells throughout the GI tract (Bach et al., 2018; Ghazanfar et al, 2017; Ghazanfar et al, 2021).

The strains used as additives in cattle feed generally are of the species *Saccharomyces cerevisiae* and were not specifically selected to be used as probiotics for cattle (Garcia-Mazcorrho et al., 2019). Despite the promising results of using *Saccharomyces cerevisiae* strains in ruminant feed, only two studies have aimed to isolate and select yeasts with probiotic potential for cattle from the gastrointestinal tract (GIT) of ruminants (Fernandes et al., 2019; Suntara et al., 2021). The probiotic capacity of microorganisms depends on their interaction with their host. To obtain consistent results, it is important to select, at the strain level, specific microorganisms for each host (FAO, 2016; Di Gioia and Biavati, 2018; Pereira et al., 2018).

Recent studies, mainly based on molecular techniques, have focussed on the ruminal environment, such as on the diversity of bacteria (Kim et al., 2017). Studies on yeast diversity and, especially, on the mode of action and the probiotic and biotechnological potential of yeasts that have been isolated from different parts of the ruminant GIT are still scarce (Fouts et al., 2012, Garcia-Mazcorro et al., 2019).

The lack of understanding of the mode of action of the different yeast strains used as food supplements for cattle makes it difficult to understand how these microorganisms benefit the host. Before testing the effects of a microorganism as a feed supplement, it is essential to know the cytological and physiological characteristics and the mode of action of each strain to determine whether the microorganism can be considered a probiotic and to ensure the efficiency and reproducibility of beneficial effects on the host (Pereira et al., 2018).

This study aimed to isolate and analyse the diversity of yeasts present in cattle faeces and to select those that have physiological and cytological characteristics that can guarantee the

probiotic potential of these microorganisms to promote the development and performance of cattle.

2 - MATERIALS AND METHODS

The experiment was performed in two stages. In the first stage, yeasts were isolated and identified from fecal samples of eleven cattle. In the second stage, the selection of yeast strains with probiotic potential for cattle was performed. In this step, the strains isolated from feces in the first step were used together with 54 strains (Supplementary Table 1) previously isolated from bovine rumen fluid samples (Fernandes et al., 2019).

2.1 – Faecal sample collection for isolation and identification of yeasts

Stool samples were collected from 11 healthy adult cattle belonging to two sample groups (Supplementary Table 2). The first group consisted of nine Holstein cows (identified as A1-A9) from the Better Nature Research Center (Ijaci, Minas Gerais, Brazil) in their lactation phase, fed a diet based on corn silage and high-energy protein concentrate. The second sample group consisted of two Nellore males weighing 320 (± 5) kg kept in Marandu grass (*Brachiaria brizantha* cv. Marandu) pastures, belonging to the Department of Animal Science of the Federal University of Lavras (Lavras, Minas Gerais, Brazil). None of the animals were given yeast supplementation.

A faecal sample was collected directly from the rectum of each animal. For Holstein cows, the collections were performed after milking, approximately 2 hours after the morning feeding. At the same time, faecal samples were collected from Nellore males that were on pasture. Twenty-five grams of each sample was homogenized separately in 225 mL of sterile 0.1% peptone water (120°C for 15 minutes). The samples were serially diluted 10-fold, spread on Petri dishes with dichloran rose-bengal chloramphenicol agar base (DRBC, HiMedia, Mumbai, India), and incubated at 28°C for 48 hours. The number of colony-forming units (CFU) was recorded, and each colony morphotype was characterized (colour, surface, elevation, and margin). Then, a number of colonies equal to the square root of the total number of colonies of each morphotype was isolated and purified using the quadrant streak method on the yeast extract peptone dextrose agar (YEPD) (g/L: yeast extract 10.0, peptone 10.0, dextrose 20.0, and agar 20.0). Each isolate was evaluated for cell morphology and mode of reproduction (Carvalho et al. 2017).

The isolates were identified and grouped statistically according to their protein profile by matrix-assisted laser desorption/ionization mass spectrometry (UltrafleXtreme MALDI-TOF MS, BrukerDaltonics, Bremen, Germany) according to Carvalho et al. (2017). Each isolate was analysed in triplicate to measure the quality and reproducibility of the spectra. The mass spectra were analysed using MALDI Biotyper 3.0 software (BrukerDaltonics). After cluster analysis of the MALDI-TOF MS spectrum, the internal transcribed spacer (ITS) region of the ribosomal DNA was sequenced to identify strains that were not identified by the MALDI-TOF MS technique. DNA was extracted by the phenol–chloroform method (Minuti et al., 2015). Polymerase chain reaction (PCR) was performed with primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), with the exception of strains of the genus *Trichosporon*, which were amplified with primers 26SF (5′-ATCCTTTGCAGACGACTTGA-3′) and 5SR (5′-AGCTTGACTTCGCAGATCGG-3′). One microlitre of template DNA was added to 15 μL of *Taq* PCR Master Mix (Qiagen, SP, Brazil), 12 μL of H_2O, and 1 μL of each primer. The amplification conditions for both primers were as follows: initial denaturation at 94°C for 3 minutes; 35 cycles at 94°C for 30 seconds, 57°C for 35 seconds, and 72°C for 1 minute; and a final extension at 72°C for 10 minutes. The amplified products were confirmed by 1% agarose gel electrophoresis. Then, the amplicons were sent for DNA sequencing at GoGenetic (Curitiba, PR, Brazil). The sequences were compared against the GenBank database using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/).

The relative frequency (%) of each species was calculated as the population of an individual species divided by the total population of yeast isolates in each of the samples analysed (Angchuan et al., 2021).

2.2 - Selection of yeasts with probiotic potential for cattle

The 139 yeasts isolated from faecal samples (in this study) and 54 yeasts isolated from ruminal fluid (Fernandes et al., 2019), deposited in the Agricultural Microbiology Culture Collection (CCMA), Department of Biology, Federal University of Lavras, Lavras, Minas Gerais, Brazil, were tested to evaluate physiological and cytological characteristics to select strains with potential for use as probiotics in cattle.

After each test, only the yeasts that showed the best results were put through the next test. Figure 1 shows the flowchart of the steps for the selection of yeasts as probiotics and the number of strains selected in each step.

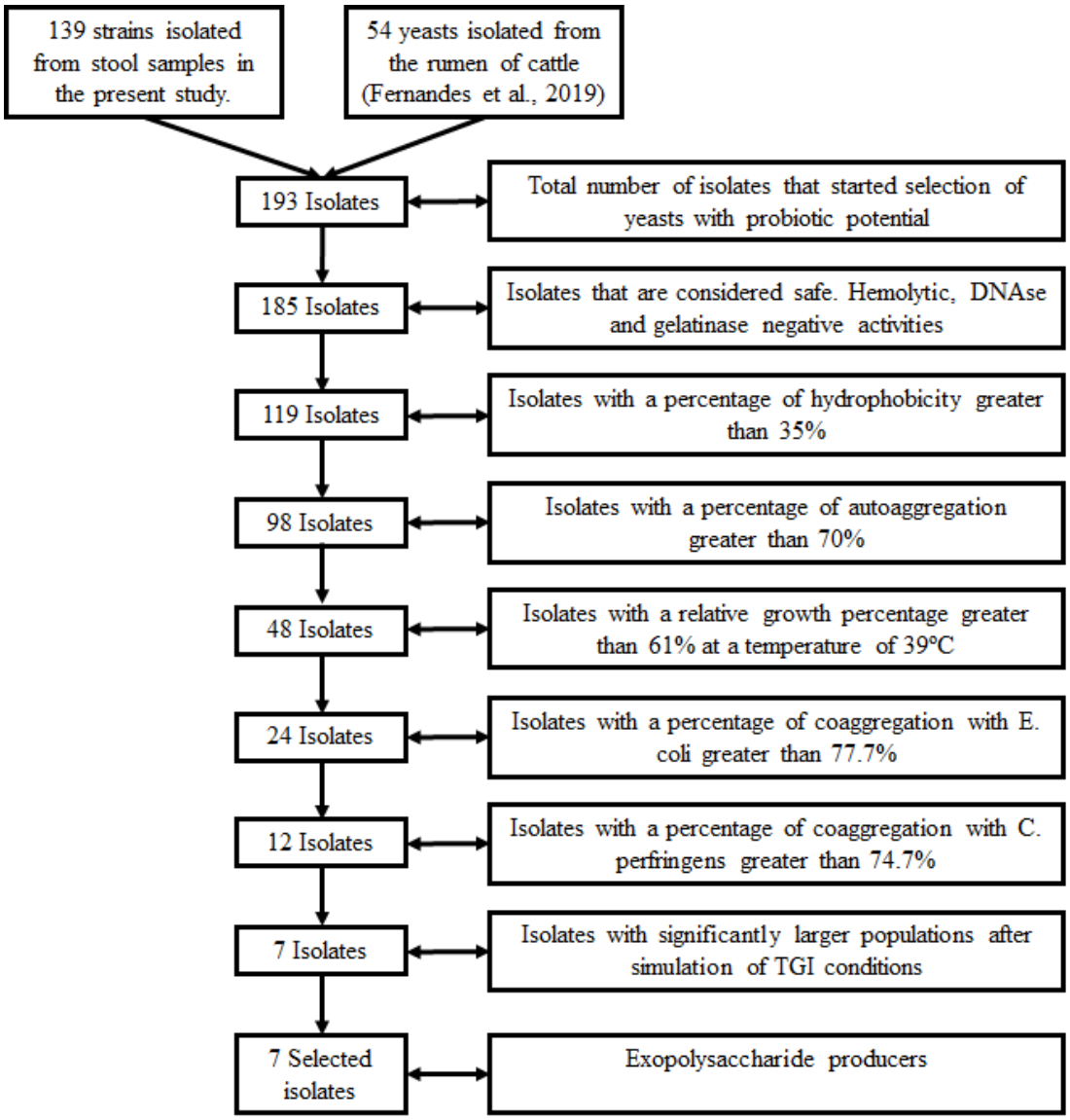


Figure 1 - Flowchart showing the number of isolates selected after each test.

2.3 - *In vitro* evaluation of safety attributes

The safety of the isolates was investigated by evaluating their haemolytic activity, DNase activity, and ability to hydrolyse gelatine. The haemolytic activity of the 193 isolates was evaluated according to Bonatsou et al. (2018), with modifications. The yeast strains were grown on blood agar base (KASVI, São José dos Pinhais, PR, Brazil) containing 5% (v/v) horse

608 blood and incubated at 28°C for 48 hours. The plates were examined for signs of β-haemolysis
609 activity (clear halos around the colonies), α-haemolysis (green halos around the colonies), and
610 γ-haemolysis (without halos around the colonies).

611 DNase production was evaluated according to Syal and Vohra (2013), with
612 modifications. The yeast isolates selected in the above assay were grown in DNase agar (Difco,
613 USA). The plates were incubated at 28°C for 48 hours, then HCl (1 N) was added to the
614 colonies. The formation of a clear halo around the colonies was considered positive for DNase
615 production.

616 The gelatinase production of the yeast isolates selected in the above tests was evaluated
617 using tryptone-neopeptone-dextrose agar (g/L: 17.0 tryptone, 3.0 neopeptone, 2.5 dextrose, 5.0
618 NaCl, 2.5 K_2HPO_4, and 20.0 agar) containing 0.4% gelatine. The Petri dishes containing the
619 medium were inoculated with the test culture and incubated at 37°C for 48 hours. After the
620 incubation period, saturated ammonium sulfate solution was added over the entire Petri dish.
621 The development of clear halos around the colonies was considered a positive reaction for the
622 production of gelatinase (Syal and Vohra, 2013).

623 The above three tests were performed in triplicate, and *Staphylococcus aureus* strain
624 ATCC25923 strain was used as a positive control.

625 **2.4- Ability to adhere to cells**

626 2.4.1 - Cell surface hydrophobicity

627 To evaluate the cell surface hydrophobicity (SHb), the selected strains were grown at
628 28°C in YEPD broth for 24 hours. The cells were separated by centrifugation at 10,000 rpm
629 and 4°C for 10 minutes. Next, the cells were washed twice and resuspended in 3 ml of
630 phosphate-buffered saline (PBS), and the absorbance ($OD_{initial}$) was measured using a
631 spectrophotometer at 600 nm. The yeast suspensions (3 ml) were placed in contact with 1 ml
632 of xylene. The cells were agitated for 120 seconds. The suspension was then kept at 37°C for
633 60 minutes to allow phase separation. After 60 minutes, the aqueous phase was carefully
634 removed, and the absorbance (OD_{final}) was measured in a spectrophotometer at 600 nm. The
635 reduction in absorbance was taken as a measure of SHb (% hydrophobicity), calculated using
636 the equation given below: % hydrophobicity = $[(OD_{initial} - OD_{final}) / OD_{initial}] \times 100$, where
637 $OD_{initial}$ and OD_{final} are the absorbances (at 600 nm) before and after extraction with

hydrocarbons, respectively (Syal and Vohra, 2013). SHb was also classified into three groups according to Colloca et al. (2000): ^{Low}SHb (<35%), ^{Moderate}SHb (35-70%), and ^{High}SHb (> 70%).

2.4.1 - Auto-aggregation assay

The auto-aggregation (AAg) assay was performed according to Syal and Vohra (2013). The yeasts were grown for 24 hours at 28°C in YEPD broth. The cells were separated by centrifugation at 10,000 rpm for 10 minutes, then washed twice and resuspended in PBS. The cell suspensions (2 mL) were agitated for 10 sec. The absorbance (A_0) was measured at 600 nm, and auto-aggregation was determined after 1 hour of incubation at 37°C. An aliquot of the upper part of the PBS suspension after incubation was transferred to another tube, and this absorbance (A_t) was measured at 600 nm. The percentage of auto-aggregation was calculated according to the following equation: $[1 - (A_t / A_0)] \times 100$, where A_t represents the absorbance at time t = 1 hour and A_0 represents the absorbance at t = 0. According to Rahman et al. (2008), the percentage of AAg of a strain can be classified into three groups: ^{low}AAg (<20%), ^{moderate}AAg (20-70%), and ^{high}AAg (> 70%).

2.5 - Growth at 28°C and 39°C

To determine the ability of the selected isolates to grow at the body temperature of cattle, the yeasts were incubated (the same initial population was used) in YEPD medium for 24 hours at two different temperatures: 28°C, the average optimal growth temperature of mesophilic yeasts that was used during isolation, and 39°C, the mean body temperature of cattle. After the incubation, the indirect growth of the yeasts was measured by reading the optical density, and the percentage of growth was calculated at 39°C ($\%C^{39}$), following the following equation: $\%C^{39} = (C^{39} \times 100) / C^{28}$, where C^{39} and C^{28} correspond to the optical densities of growth at 39°C and 28°C, respectively.

2.6 - Coaggregation with pathogens

The yeast isolates selected in the previous assay were grown for 24 hours at 28°C in YEPD broth. The cells were separated by centrifugation at 10,000 rpm for 10 minutes, then washed twice and resuspended in PBS to $OD_{600} \approx 0.5$. Equal volumes (1 ml) of the pathogenic bacteria [*Escherichia coli* (CDC 055 - EPEC) and *Clostridium perfringens* (ATCC 1803)] were individually mixed with the evaluated yeasts and incubated at 37°C for 2 hours without agitation. Next, the absorbance of the mixture (OD_{mix}) described above and of the yeast

(OD_{yeasts}) and pathogen suspensions ($OD_{pathogens}$) alone were measured at 600 nm. Coaggregation was calculated according to the following equation: %Coaggregation = [1 − [OD_{mix} / (OD_{yeast} + $OD_{pathogen}$) / 2]] × 100. The assay was performed first by evaluating coaggregation with *E. coli*. The isolates that showed coaggregation results with *E. coli* higher than the mean value were then tested for their ability to coaggregate with *C. perfringens*.

2.7 - Simulation of viability under GIT conditions

The viability of the selected yeasts was tested under conditions based on the characteristics of the GIT of cattle (Colucci et al., 1986; Mambrini and Peyraud, 1996; Kung et al., 1997; Ogunremi et al., 2015). Sterile ruminal fluid was used as the substrate for this evaluation. Samples of ruminal fluid were collected from cannulated Zebu heifers 2 hours after feeding and autoclaved for 15 minutes. The yeasts tested were grown for 24 hours in YEPD medium, and an initial population of 6.0 log CFU/mL (determined by counting in a Neubauer chamber) was inoculated in 50 ml of ruminal fluid. The ruminal fluid was incubated for 27 hours at 39°C with 100 rpm agitation. The pH of the ruminal fluid was reduced to 2 using HCl 1 M, pepsin (3.0 g/L) was added and incubated for 6 hours at 39°C and 100 rpm. Then, the pH was adjusted to 8 with $NaHCO_3$ 1 M, and pancreatin (1.0 g/L) and bile salts (3.0 g/L) were added before another incubation of 12 hours at 39°C and 100 rpm. Last, the population of viable cells was evaluated by surface plating in DRBC medium. The assay was performed in triplicate.

3 - RESULTS

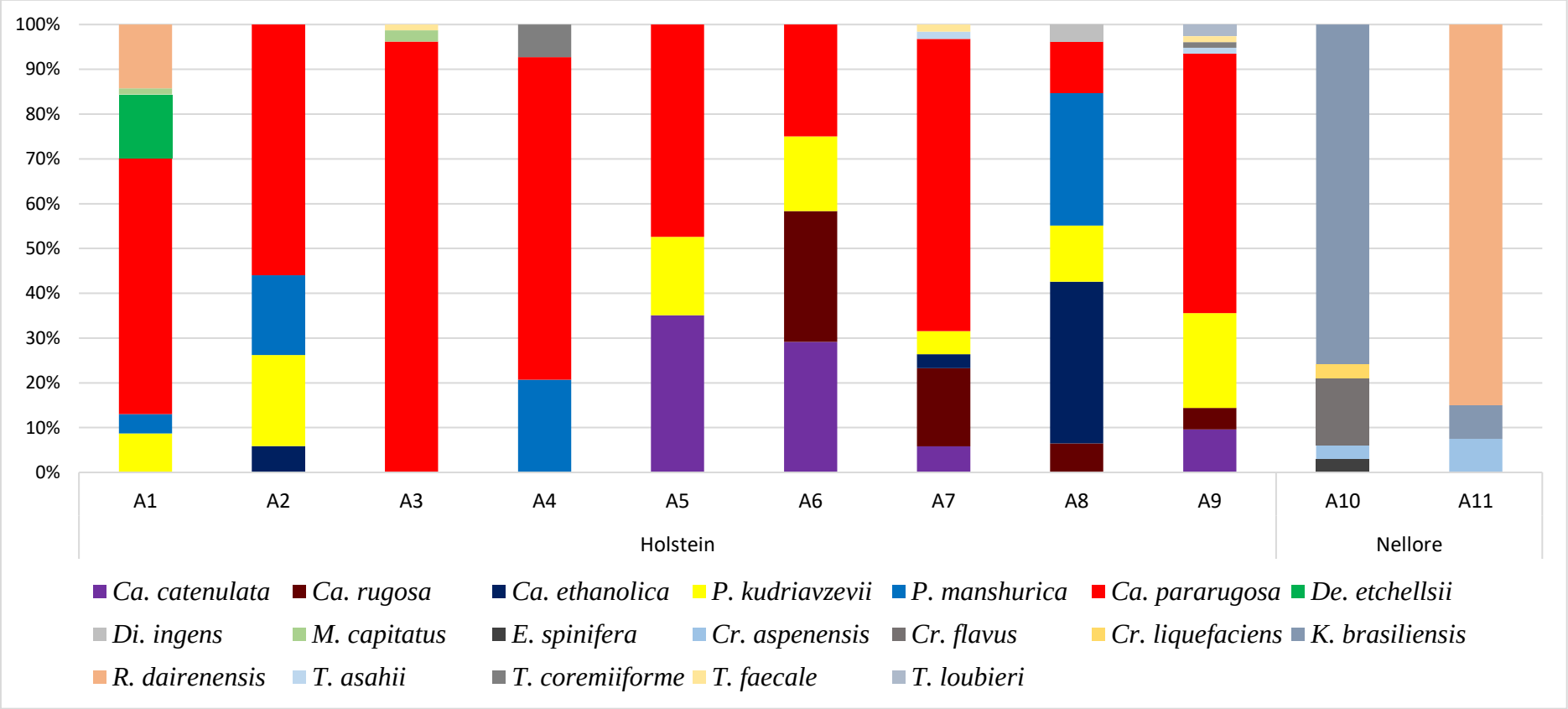
3.1 - Isolation and identification of faecal yeasts

A total of 139 yeasts were isolated from the faeces of cattle (Supplementary Table 1), belonging to 10 genera and 19 different species of the phyla Ascomycota and Basidiomycota (Figure 2 and Supplementary Table 3): *Candida catenulata* (10 isolates), *Candida ethanolica* (7), *Candida pararugosa* (44), *Candida rugosa* (8), *Cryptococcus aspenensis* (2), *Cryptococcus flavus* (1), *Cryptococcus liquefaciens* (1), *Debaryomyces etchellsii* (1), *Dipodascus ingens* (2), *Exophiala spinifera* (1), *Pichia kudriavzevii* (24), *Kalmanozyma brasiliensis* (6), *Magnusiomyces capitatus* (3), *Pichia manshurica* (12), *Rhodotorula dairenensis* (7), *Trichosporon asahii* (2), *Trichosporon coremiiforme* (3), *Trichosporon faecale* (3), and *Trichosporon loubieri* (2). In the samples where we could detect a viable population, the yeast

697 population varied from 3.51 to 4.99 log CFU/mL, and among all the identified species, the
698 species *Ca. pararugosa* had the largest population (4.89 log CFU/mL), in sample A3
699 (Supplementary Table 2).

700 The species *Ca. pararugosa* had a higher relative frequency in seven of the nine
701 samples collected from Holstein cows, representing 54.2% of the microorganisms isolated from
702 these samples. In Holstein cows, the species *P. kudriavzevii* and *Ca. catenulata* were the species
703 with the second- and third-highest mean relative frequencies, representing 11.3% and 8.9%,
704 respectively. The species *Ca. rugosa* and *Ca. catenulata* had the highest relative frequency
705 (29.2% each) in sample A6, while the species *Ca. ethanolica* was the species with the highest
706 relative frequency (36.1%) in sample A8. In the samples collected from Nellore males, *K.*
707 *brasiliensis* was the species with the highest relative frequency (75.8%) in sample A10, and *R.*
708 *dairenensis* was the one with the highest relative frequency (85.0%) in sample A11 (Figure 2
709 and Supplementary Table 3).

710 The species *Ca. pararugosa* was the species with the highest occurrence, isolated from
711 all samples collected from Holstein females, and was therefore present in nine of the eleven
712 samples analysed. The species with the second-highest occurrence was *P. kudriavzevii*, isolated
713 from seven samples. The species *R. dairenensis* was the only species isolated from the samples
714 of Holstein females and from samples of Nellore males. *Ca. ethanolica*, *Ca. pararugosa*, *Ca.*
715 *rugosa*, *De. etchellsii*, *Di. ingens*, *P. kudriavzevii*, *M. capitatus*, *P. manshurica*, *T. asahii*, *T.*
716 *coremiiforme*, *T. faecale*, and *T. loubieri* were identified only in the samples of Holstein
717 females. In addition, *Cr. aspenensis*, *Cr. flavus*, *Cr. liquefaciens*, *E. spinifera*, and *K.*
718 *brasiliensis* were isolated only from samples of Nellore males (Figure 2 and Supplementary
719 Table 3).



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721 **Figure 2**- Relative abundance (%) of yeast species identified from bovine fecal samples.

3.2 - Safety assessment

Only one isolate, of the species *Ca. pararugosa* (L21), showed β-haemolytic activity. None of the isolates had a positive gelatinase test. Seven isolates (3.6% of the total)—five of *Ca. ethanolica*, one of *Ca. rugosa*, and one of *P. manshurica*—showed DNase activity. The isolates that were positive for the haemolytic and DNA activity tests were eliminated, and the others were put through subsequent tests (Table 1). A total of 185 isolates were selected for the next test.

Table 1 - Safety test results and percentage of isolates with a positive result relative to the total number of isolates.

Tests	Strains	Percentage (%)[1]
Haemolytic activity	L21 -*Candida pararugosa*	0,52
DNase activity	L14 -*Candida ethanolica* L15 -*Candida ethanolica* L16 -*Candida ethanolica* L12 -*Candida ethanolica* L13 -*Candida ethanolica* L69 -*Candida rugosa* L121 -*Pichia manshurica*	3,6
Hydrolyse gelatine	Nenhum resultado positivo	0
Safety attributes	185 cepas	95,8

1 - Percentage of isolates with a positive test result relative to the total number of isolates.

3.3 - Cell adhesion ability

The SHb values of the isolates ranged from 2.8% (L34 - *Ca. pararugosa*) to 92.8% (L136 - *T. faecale*). Of these, 66 isolates (35.7%) had a low percentage of SHb, 93 (50.3%) had moderate SHb and 26 (14.0%) had a high percentage of SHb. The isolates with low SHb (below 35%) were eliminated from the selection process (Table 2). A total of 119 isolates remained for the next test.

The isolates showed AAg capacity ranging from 32.8% (L80 - *K. brasiliensis*) to 98.6% (CCMA 913 - *P. kudriavzevii*). None of the isolates had a low percentage of AAg; 21 isolates (17.6%) had moderate AAg capacity, and 98 (83.4%) had high AAg capacity. Only the isolates with high AAg capacity were kept for the next tests (Table 2), totalling 98.

Table 2 - Ability to adhere to the cell surface. Results of hydrophobicity and autoaggregation tests.

Test		Strains	Percentage (%)[1]	Selection
Hydrophobicity	Low (<35%)	66	35,7	Excluded
	Moderate (35–70%)	93	50,3	Selected
	High (>70%)	26	14,0	
Auto-aggregation	Low (<20%)	0	0	Excluded
	Moderate (20–70%)	21	17,6	
	High (> 70%)	98	83,4	Selected

1- Percentage of isolates in relation to the total amount analyzed.

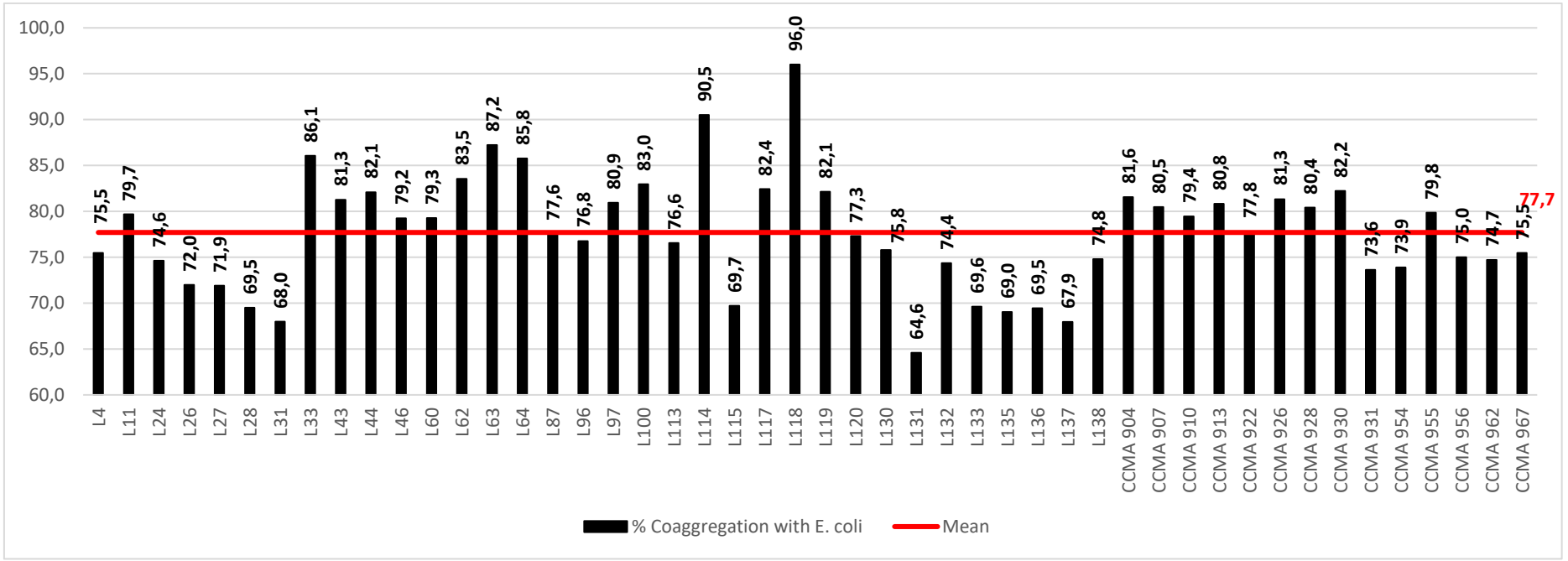
3.4 - Growth at 28°C and 39°C

All yeasts had the ability to grow at a temperature equivalent to the average body temperature of cattle, though all showed lower growth at 39°C than at 28°C. The growth percentage at 39°C ranged from 13.0% (CCMA 948 - *P. manshurica*) to 95.6% (L131 - *T. asahii*) compared to growth at 28°C. The mean relative growth at 39°C was 61%, and the 50 isolates that showed a percentage growth below the mean did not proceed to the next tests. A total of 48 isolates were selected for the next test.

3.5 - Inhibitory activity against enteric pathogens

All 48 isolates analysed could coaggregate with *E. coli* (CDC 055 - EPEC). This capacity ranged from 64.6% (L131 - *T. asahii*) to 96.0% (L118 - *P. manshurica*). The mean percentage of coaggregation with *E. coli* was 77.7%, and the 24 isolates that presented a lower percentage than the mean did not proceed to the next tests (Figure 3). A total of 24 isolates were kept for the next assay.

All isolates analysed showed the ability to coaggregate with C. *perfringens* (ATCC 1803), and the coaggregation capacity ranged from 48.9% (CCMA 910 - *P. kudriavzevii*) to 85.4% (L64 - *Ca. rugosa*). The mean percentage of coaggregation with *C. perfringens* was 75.4%. The 12 isolates that had a lower percentage than the mean did not proceed to the next test (Figure 4), so 12 isolates went on to the next test.



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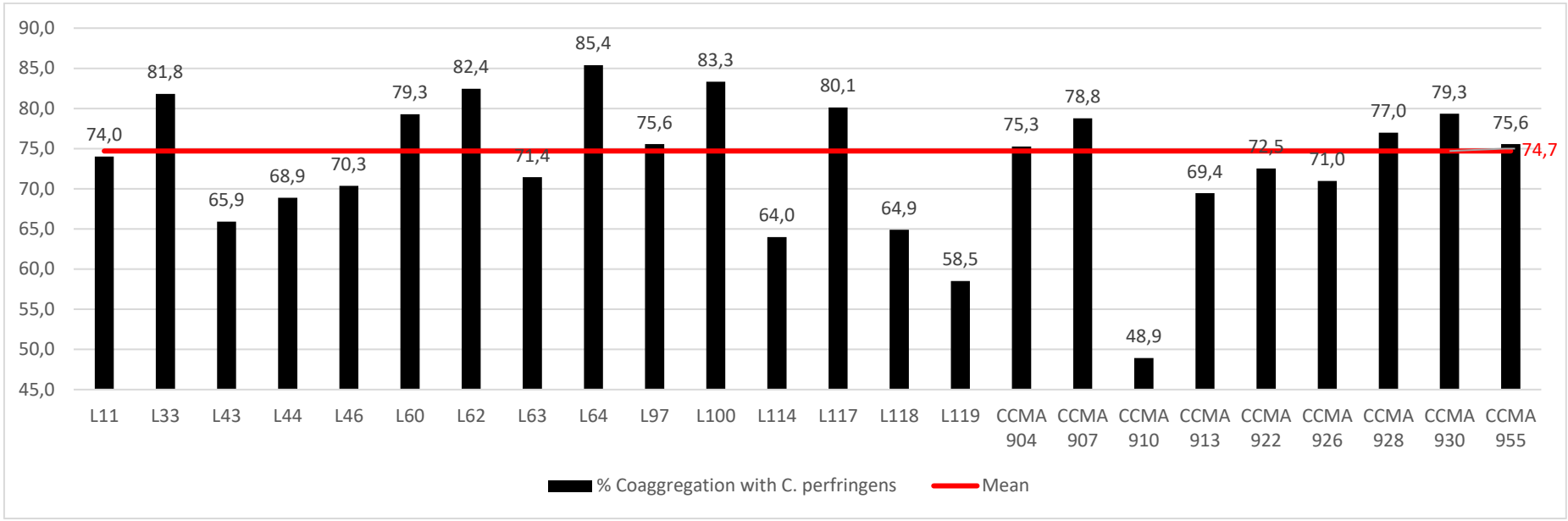
765 **Figure 3 -** Percentage of coaggregation of tested strains with *Escherichia coli* (CDC 055 - EPEC).



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767 **Figure 4 -** Percentage of coaggregation of tested strains with *Clostridium perfringens* (ATCC 1803).

768 **3.6 - Viability under GIT conditions**

769 The 12 strains selected by the previous tests showed, at the end of the evaluation of
770 viability under cattle GIT conditions, a final population that ranged from 3.4 (L33 - *Ca.*
771 *pararugosa*) to 6.3 (CCMA 928 - *Ca. pararugosa*) log CFU/ml (Table 3). Seven strains were
772 selected that had a final population significantly higher than the others, above 5.78 log CFU/mL
773 (Table 4). These strains belonged to two different species, *Ca. pararugosa* (isolates L60,
774 CCMA 928 and CCMA 930), and *P. kudriavzevii* (isolates L97, L100, CCMA904, CCMA
775 907)). Among the selected strains, three were isolated from faeces (L60, L97 and L100), and
776 four were isolated from ruminal fluid (CCMA 907, CCMA 928, CCMA 930 and CCMA 955).

777 **Table 3-** Final population (log CFU/ml) of isolates evaluated under TGI conditions.

Strains	Mean values[1]
L33 -*Candida pararugosa*	3,41[c]
L60 – *Candida pararugosa*	5,78[a]
L62 - *Candida rugosa*	3,93[c]
L64 - *Candida rugosa*	3,71[c]
L97 – *Pichia kudriavzevii*	6,29[a]
L100 – *Pichia kudriavzevii*	6,11[a]
L117 – *Pichia manshurica*	4,63[b]
CCMA 904 – *Pichia kudriavzevii*	6,29[a]
CCMA 907 – *Pichia kudriavzevii*	6,31[a]
CCMA 928 – *Candida pararugosa*	6,29[a]
CCMA 930 – *Candida pararugosa*	6,14[a]
CCMA 955 - *Candida rugosa*	5,01[b]
SEM	0,36
P	<0,01

778 1 - Mean values in columns with different letters are statistically different (P < 0.05) based on Scott-
779 Knott test at 5% probability

3.7 - Production of exopolysaccharides

All seven yeasts selected had a positive result for the production of exopolysaccharides, showing white colonies after being grown in medium containing milk and ruthenium red.

3.8 - Correlation analysis

From the principal component analysis (Figure 5), we observed that there was a positive correlation between the AAg result and the survival at the GIT temperature. The strains L97, CCMA 904, CCMA 907, and CCMA 928 were more correlated with the SHb, AAg, and survival capacity in the GIT. The L60 strain was correlated with the growth capacity at 39°C. The CCMA 930 strain was correlated with coaggregation with *E. coli* and *C. perfringens*.

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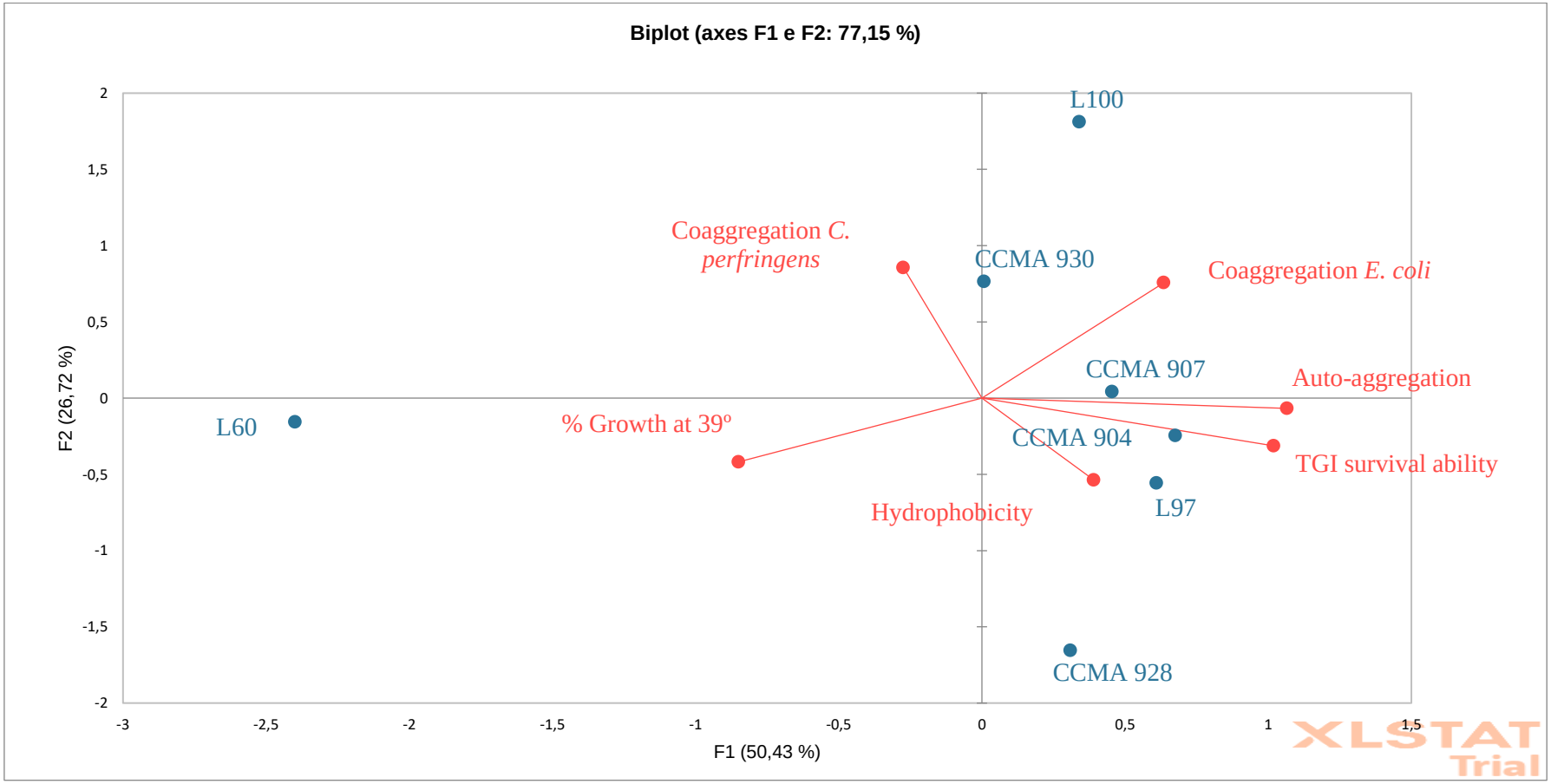

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792 **Figure 5-** Principal component analysis (PCA) based on the correlation between the yeasts selected as potential probiotics and
793 the selection tests.

4 - DISCUSSION

The diversity of yeasts presents in the gastrointestinal tract of cattle, especially in the post ruminal GIT, is still little known, and in the present study, 139 yeasts belonging to 19 species were isolated and identified.

Isolated yeasts belonging to the genera *Pichia*, *Candida*, and *Debaryomyces* have been previously described because they have probiotic properties that lead to health benefits for monogastric animals (Nayak, 2011; Vohra et al., 2016) and are among the main fungi found in the GIT of humans (Hallen-Adams et al., 2015). There are no reports of the identification and isolation of *Cr. aspenensis*, *Cr. flavus*, *Cr. liquefaciens*, *E. spinifera*, or *T. loubieri* in the gastrointestinal tract of cattle, humans, or other animals. There is no consensus on the dominance of a particular yeast species in cattle GIT, but the published studies suggest that there is a predominance of species of the genus *Candida* (*Ca. albicans*, *Ca. parapsilosis*, *Ca. tropicalis*, *Ca. rugosa*) throughout the GIT and of *P. kudriavzevii* in samples of rumen content (Van Under and Souza, 1957; Clarke and Menna 1961; Lund, 1974; Lund 1980; Fernandes et al., 2019; Angchuan et al., 2021). Among the identified yeasts, strains belonging to the species *Ca. ethanolica*, *Ca. pararugosa*, *Ca. rugosa*, and *P. kudriavzevii* have already been described because they have probiotic potential for cattle (Fernandes et al., 2019; Suntara et al., 2021). Strains of the species *P. kudriavzevii* stand out because they have been isolated from different substrates and have potential for use as probiotics for humans (Chelliah et al., 2016; Greppi et al., 2017; Saadat et al., 2020).

No yeasts belonging to the genus *Saccharomyces* were isolated from the analysed samples. Other studies that isolated and identified yeasts from the bovine GIT also did not observe the presence of *Saccharomyces* (Lund, 1974; Lund, 1980; Fernandes et al., 2019; Angchuan et al., 2021; Suntara et al., 2021). *Saccharomyces* is the most commonly used probiotic yeasts for humans (Czerucka et al., 2007) and cattle (Garcia-Mazcapse et al., 2019). A probiotic strain can be isolated from different substrates to exert its benefits in different animals, and it is not necessary to use a strain that is autochthonous to the host (Garcia-Mazcapse et al., 2019). However, Ghazanfar (2021) compared the effect of supplementation with autochthonous yeast from the GIT of dairy cows and allochthonous yeasts and concluded that autochthonous yeasts can better adapt to the intestinal conditions of the animals from which they were isolated when compared with yeasts isolated from another ecosystem. In addition,

825 the authors observed that indigenous yeasts showed better results for animal health and welfare,
826 improving the production of digestive enzymes and the equilibrium of the ruminal microbiota
827 by stimulating beneficial microorganisms (*Pediococcus* and *Weissella*) and inhibiting the
828 growth of pathogens (*E. coli*).

829 Only the species *R. dairenensis* was isolated both from the faecal samples of dairy
830 cows that consumed silage and from the Nellore males that grazed. The objective of the present
831 study was not to compare the diversity of yeasts in the faeces of animals fed silage with animals
832 on pasture, but this information is important, as the GIT microbiota is associated with the diet,
833 breed, age, and physiological stage of the animals (Petri et al., 2012; Loor et al., 2016; Sales et
834 al., 2021). In the study by Lund (1974), animals consuming silage had a larger yeast population
835 than animals consuming fresh grasses. Further studies are needed to determine whether there is
836 an association between the yeasts present in the silage provided and the yeasts present in the
837 GIT of ruminants.

838 Most studies on yeast diversity in the bovine GIT have focussed on evaluating samples
839 of rumen content. Only two studies were found that isolated and identified yeasts from the
840 intestines of cattle (Van Under and Souza, 1957; Angchuan et al., 2021). Van Under and Souza
841 (1957) evaluated yeasts present in the caecum of 252 adult cattle and identified 17 different
842 species. Among them, *Ca. tropicalis* was the most abundant species, followed by *P.*
843 *kudriavzevii*, the only species they found that we isolated in the present study. Angchuan et al.
844 (2021) isolated 1126 yeasts from different regions of the small intestine (duodenum, jejunum,
845 ileum). *Ca. parapsilosis* showed a higher frequency of occurrence in the liquid and epithelium
846 samples. The authors observed that there was no difference between the yeast community
847 belonging to the liquid and to the epithelium of the samples collected from the duodenum,
848 jejunum, or ileum. The species *Ca. ethanolica*, *Ca. pararugosa*, *Ca. rugosa*, *De. etchellsii*, *P.*
849 *kudriavzevii*, *P. manshurica*, and *T. asahii* were the species they isolated that we found in the
850 present study.

851 After identification of the isolates, we performed the safety test first so that no strain
852 with pathogenic potential could proceed to the next stage of selection. After being subjected to
853 tests that evaluated three different modes of pathogenic action, ability to lyse red blood cells
854 (haemolysis), production of the deoxyribonuclease enzyme, and production of the proteolytic
855 enzyme gelatinase (virulence factor associated with the ability to hydrolyse collagen,

haemoglobin, and other bioactive peptides), 95.9% of the isolates showed negative results in all safety tests and were considered safe as probiotic candidates. Some of the isolated species, *Ca. catenulata*, *Ca. rugosa*, *Cr. flavus*, *P. kudriavzevii* (*Ca. kruzei*), *T. coremiiforme*, and *T. asahii*, have been reported as opportunistic pathogens in cattle because they have been isolated from animals with mastitis (Cabañes, 2009; Refai and El-Yazid, 2017). Although identification at the species level is important to obtain preliminary information on microorganisms, strains of the same species may or may not have pathogenic potential, which emphasizes the importance of selecting probiotics at the strain level and performing safety tests regardless of the identity assigned (Shanahan, 2012; Pereira et al., 2018).

On the safety of using yeasts as probiotics, an advantage they have over bacteria is that yeasts are eukaryotes, so horizontal gene transfer occurs less frequently than in prokaryotes, and the transfer of antibiotic resistance genes is considered one of the main concerns regarding the safety of prokaryotic microorganisms used as probiotics (Shanahan, 2012). A characteristic of probiotic yeasts that have been commercialized is that they can be used during treatment with antibiotics (Nayak, 2011), an important characteristic that can assist in the management of cattle.

Hydrophobicity and auto-aggregation assays were performed to evaluate the ability of probiotic candidates to colonize GIT epithelial cells, an essential characteristic of a probiotic. The review by Pereira et al. (2018) shows that an SHb above 30-40% is sufficient for yeast to adhere to GIT cells. Thus, yeasts with an SHb above 35% were selected. The hydrophobicity of the cell surface allows a better interaction between the microorganism and the epithelial cells of the GIT (Duary et al., 2011). Yeasts may have an advantage in the elimination of pathogenic bacteria through a mechanism of competition for space, since the cells of yeasts are usually larger than bacteria, so a single yeast cell needs a larger space to adhere to the surface of an intestinal cell than a bacterial cell (Nayak, 2011).

No reference was found on the ideal AAg value, and the strains with the highest AAg capacity (^{High}AAg> 70%) after 1 hour were selected. The ability of microorganisms to auto-aggregate ensures that the probiotic reaches high cell density in the intestine, contributing to the adhesion mechanism (Pereira et al., 2018). The PCA showed a positive correlation between AAg and survival under the GIT conditions. This correlation indicates that yeasts with a greater ability to form cellular aggregates, in addition to having greater chances of adhering to intestinal

mucosa cells, as described in the literature (Pereira et al., 2017), also have a greater ability to survive under different conditions found in the GIT of cattle.

Competition for space seems to be the main mode of action by which the selected strains inhibit the growth of pathogenic microorganisms. In addition to their ability to adhere to GIT epithelial cells, the microorganisms selected had a greater ability to coaggregate with *E. coli* and *C. perfringens*, two species of microorganisms considered to be the main causes of diarrhoea and enterotoxaemia in calves and adult cattle, respectively (Lebrun et al., 2010; Blanchard, 2012). According to Suarez and Guevara (2018), the presence of live yeast in the digestive tract of animals causes a phenomenon called competitive exclusion, in which certain pathogenic bacteria adhere to the surface of the yeast, thus removing a significant amount of the population of undesirable microorganisms. Certain pathogens, such as *E. coli*, have mannose-specific fimbriae (type 1) that bind to mannose residues of the intestinal epithelial cell membranes. However, these pathogens can bind to the outer layer of the yeast cell wall, which has mannoproteins on its surface, instead of binding to intestinal cells, and be eliminated from the GIT of the host after being agglutinated by the yeast (Nayak, 2011). These data show the possibility of reducing the use of antibiotics in cattle management by using probiotic strains to reduce infection by pathogenic microorganisms.

Another important mode of action of probiotics is the ability to modulate the host immune response. For this to occur with greater efficiency, probiotic microorganisms must be able to remain intact after passing through the host's GIT and to interact with the epithelial cells of the rumen and intestine (Rigobelo and Ávila, 2012; Dias et al., 2017; Ghazanfar et al., 2017; Ma et al., 2018). Cattle are ruminants and therefore have specific physiological characteristics that are different from those of humans and other monogastric animals (Hofmann, 1989). Strains with the highest viability were selected under conditions specific to these animals. In the present study, strains that showed higher relative growth at 39°C (mean body temperature of cattle) were selected, and this temperature was also used in the tests that simulated the GIT. The ability to tolerate and grow at the host body temperature is a key characteristic for their probiotic potential to remain viable throughout the GIT. In the study by Lund (1974), the population of yeasts present in the ruminal fluid of cattle was equal to 1.3×10^5 cells/mL when incubated at 25°C, but the population count dropped to 3.5×10^3 cells/mL when the ruminal fluid was incubated at 39°C, demonstrating that some yeasts present in the ruminal fluid could not grow at the mean body temperature of the cattle.

We simulated the GIT to evaluate the viability of the isolates under these conditions, and ruminal fluid was used as the growth medium. The pH values were based on the rumen (pH 7.0), abomasum (pH 2.0), and intestine (pH 8.0) (Harfoot, 1981), and the time spent at each of these pH values was estimated based on the passage rate and retention time in each of these GIT regions (Colucci et al., 1986; Mambrini and Peyraud, 1996). To evaluate viability after traversing the entire GIT, the test was performed continuously. The selected strains had a high viability rate (> 96.7%), with a final viable population very close to the initial population. Some strains could grow under GIT conditions and showed final populations larger than the initial population at the end of all simulation stages. One of the factors that may explain these high values is that the strains were isolated from the cattle GIT itself (three strains isolated from the faeces and four strains isolated from the rumen) and are probably adapted to the different conditions of this ecosystem. Other authors isolated yeasts from ruminal fluid, and they observed that yeasts can remain viable for long periods (48 hours) under ruminal conditions (Kung et al., 1997; Fernandes et al., 2019). The species isolated and identified in ruminal environments are mostly similar to those that this study isolated and identified in the intestine (Lund, 1994; Lund, 1980; Fernandes et al., 2019; Suntara et al., 2021). This similarity helps to emphasize that although they are considered transient inhabitants of the rumen and are not numerous in the GIT of cattle, yeasts are present and may remain metabolically active in the rumen and remain viable after passing through the GIT.

The selected yeasts produce exopolysaccharides (EPSs). EPSs are polymers of high-molecular-weight monosaccharides produced by different species of microorganisms to protect themselves against desiccation, bacteriophages, antibiotics, and osmotic stress; to adhere to solid surfaces and form biofilms; and for cell recognition. EPSs are produced for longer in the gastrointestinal tract, an important characteristic for interaction with the host and for action against pathogen infection, since they might make yeast colonization more efficient. Microorganisms producing EPSs can be used as immunostimulants because EPSs play an important role in the activation of macrophages and lymphocytes. Bach et al. (2018) observed that supplementation with viable yeasts could stimulate the production of genes related to the immune response of cattle. In the rumen, the expression of genes (tumour necrosis factor α, interleukin 10, proliferating cell nuclear antigen, and Ki-67 antigen) that regulate inflammation and the epithelial barrier increased after supplementation of animals with yeasts. In the colon,

yeast supplementation increased the expression of the β-defensin gene, which encodes an antimicrobial peptide that can augment the host immune response.

The seven strains selected belonged to two different species, *Ca. pararugosa* (isolates L60, CCMA 928, CCMA 930) and *P. kudriavzevii* (isolates L97, L100, CCMA904, CCMA 907). *Ca. pararugosa* was first described by Nakase et al. (1979) when it was isolated from human faeces and has since been described as part of the human GIT microbiota (Suzuki et al., 1999). Paredes et al. (2012) isolated strains of *Ca. pararugosa* from different anatomical regions, such as saliva, bronchi, blood, urine, and the vagina, from humans and other species. Fernandes et al. (2019) identified strains belonging to this species in samples of the rumen content of cattle. One strain (CCMA 970) of *Ca. pararugosa* was among the strains that showed greater survival capacity in the ruminal fluid, in addition to stimulating the production of organic acids in the rumen, and was defined as having probiotic potential for cattle (Fernandes et al., 2019). These results, combined with the probiotic characteristics observed in the present study, show that strains belonging to this species have great potential as probiotics for cattle and may have beneficial actions both in the rumen and in the intestine.

P. kudriavzevii has three other heterotypic synonyms, *Candida krusei*, *Issatchenkia orientalis*, and *Candida glycerinogenes* (Douglass et al., 2018). The name *Candida krusei* is used in research associated with candidiasis, and this species is responsible for approximately 2% of fungal infections caused by *Candida* species in humans (Pfaller et al., 2017). The name *Pichia kudriavzevii* is used by industry and is associated with research in the food industry. Despite being associated with infections, this yeast is not considered a pathogen. It received the status of generally recognized as safe by the United States Food and Drug Administration because it is often isolated from spontaneous fermentations of cassava, cocoa, and milk (Bourdichon et al., 2012). This species is used as a starter culture for several industrial products because it has probiotic potential for humans, such as resistance to physiological concentrations of bile salts, pepsin, and pancreatic enzymes, efficient auto-aggregation, coaggregation capacity with commercial probiotic yeasts, and good hydrophobicity. In addition, a strain belonging to this species inhibits the growth of 13 enteropathogens (Chelliah et al., 2016).

Santos et al. (2015) concluded that the addition of *P. kudriavzevii*, despite reducing the *in vitro* digestion of neutral detergent fibre, altered other aspects of ruminal fermentation, thus increasing the concentration of volatile fatty acids in the rumen, which is a desirable

characteristic for a cattle probiotic. In the studies by Lund (1974), Fernandes et al. (2019), and Suntara et al. (2021), the species *P. kudriavzevii* was the one with the highest population in the ruminal fluid of cattle. Suntara et al. (2021) isolated strains of the species P. kudriavzevii from the rumen fluid of dairy cows and selected one strain (H-KKU20) as a probiotic candidate for cattle because of its high capacity to produce biomass and cellulolytic enzymes. In the present study, the population of yeasts belonging to this species ranged from 2.52 to 4.20 log CFU/mL and was the second most frequent species. The fact that this species occurs in a relatively high population in the rumen and intestine of cattle indicates that it is adapted to the GIT of these animals, a desirable characteristic that may help in the selection of probiotic strains and the effectiveness of their beneficial functions for the host.

The tests performed focused on the selection of physiological and cytological characteristics of the strains to ensure the probiotic potential of the selected yeasts and the reproducibility of the positive results for the host. The seven selected strains showed the best results and guaranteed the ability to survive the conditions of TGI and interact with the cells of the intestinal mucosa, being able to inhibit the colonization of pathogens and stimulate the host immune system. The selected strains have similar probiotic potential, making it difficult to single out just one strain among them. However, principal component analysis shows that the strains have results that are correlated with different potentials and can be used specifically or together to enhance the probiotic mode of action, depending on the expected benefits.

5 - CONCLUSION

In this study, 139 strains belonging to 19 different yeast species were isolated and identified in bovine fecal samples. Strains with specific physiological and cytological characteristics were selected to be used as probiotics for cattle. The selected strains were able to survive in the conditions of GIT these animals and exhibited characteristics that allow interaction with the host intestine to prevent infections by pathogens and stimulate the immune system. Among the species analyzed, the strains of Candida paraugosa (L60, CCMA 928 and CCMA 930) and Pichia kudriavzevii (L97, L100, CCMA904, CCMA 907) stood out and can be considered as potential probiotics for use as feed additives for cattle.

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Table Supplementary 1 - Identification of yeast strains isolated from bovine intestine and rumen fluid from culture collection (CCMA).

Codes	Identification
L1	Candida catenulata
L2	Candida catenulata
L3	Candida catenulata
L4	Candida catenulata
L5	Candida catenulata
L6	Candida catenulata
L7	Candida catenulata
L8	Candida catenulata
L9	Candida catenulata
L10	Candida catenulata
L11	Candida ethanolica
L12	Candida ethanolica
L13	Candida ethanolica
L14	Candida ethanolica
L15	Candida ethanolica
L16	Candida ethanolica
L17	Candida ethanolica
L18	Candida pararugosa
L19	Candida pararugosa
L20	Candida pararugosa
L21	Candida pararugosa
L22	Candida pararugosa
L23	Candida pararugosa
L24	Candida pararugosa
L25	Candida pararugosa
L26	Candida pararugosa
L27	Candida pararugosa
L28	Candida pararugosa

Codes	Identification
L29	Candida pararugosa
L30	Candida pararugosa
L31	Candida pararugosa
L32	Candida pararugosa
L33	Candida pararugosa
L34	Candida pararugosa
L35	Candida pararugosa
L36	Candida pararugosa
L37	Candida pararugosa
L38	Candida pararugosa
L39	Candida pararugosa
L40	Candida pararugosa
L41	Candida pararugosa
L42	Candida pararugosa
L43	Candida pararugosa
L44	Candida pararugosa
L45	Candida pararugosa
L46	Candida pararugosa
L47	Candida pararugosa
L48	Candida pararugosa
L49	Candida pararugosa
L50	Candida pararugosa
L51	Candida pararugosa
L52	Candida pararugosa
L53	Candida pararugosa
L54	Candida pararugosa
L55	Candida pararugosa
L56	Candida pararugosa

Codes	Identification
L57	Candida pararugosa
L58	Candida pararugosa
L59	Candida pararugosa
L60	Candida pararugosa
L61	Candida pararugosa
L62	Candida rugosa
L63	Candida rugosa
L64	Candida rugosa
L65	Candida rugosa
L66	Candida rugosa
L67	Candida rugosa
L68	Candida rugosa
L69	Candida rugosa
L70	Cryptococcus aspenensis
L71	Cryptococcus aspenensis
L72	Cryptococcus flavus
L73	Cryptococcus liquefaciens
L74	Debaryomyces etchellsii
L75	Dipodascus ingens
L76	Dipodascus ingens
L77	Exophiala spinifera
L78	Kalmanozyma brasiliensis
L79	Kalmanozyma brasiliensis
L80	Kalmanozyma brasiliensis
L81	Kalmanozyma brasiliensis
L82	Kalmanozyma brasiliensis
L83	Kalmanozyma brasiliensis
L84	Magnusiomyces capitatus
L85	Magnusiomyces capitatus
L86	Magnusiomyces capitatus

Codes	Identification
L87	Pichia kudriavzevii
L88	Pichia kudriavzevii
L89	Pichia kudriavzevii
L90	Pichia kudriavzevii
L91	Pichia kudriavzevii
L92	Pichia kudriavzevii
L93	Pichia kudriavzevii
L94	Pichia kudriavzevii
L95	Pichia kudriavzevii
L96	Pichia kudriavzevii
L97	Pichia kudriavzevii
L98	Pichia kudriavzevii
L99	Pichia kudriavzevii
L100	Pichia kudriavzevii
L101	Pichia kudriavzevii
L102	Pichia kudriavzevii
L103	Pichia kudriavzevii
L104	Pichia kudriavzevii
L105	Pichia kudriavzevii
L106	Pichia kudriavzevii
L107	Pichia kudriavzevii
L108	Pichia kudriavzevii
L109	Pichia kudriavzevii
L110	Pichia kudriavzevii
L111	Pichia manshurica
L112	Pichia manshurica
L113	Pichia manshurica
L114	Pichia manshurica
L115	Pichia manshurica
L116	Pichia manshurica

Codes	Identification
L117	Pichia manshurica
L118	Pichia manshurica
L119	Pichia manshurica
L120	Pichia manshurica
L121	Pichia manshurica
L122	Pichia manshurica
L123	Rhodotorula dairenensis
L124	Rhodotorula dairenensis
L125	Rhodotorula dairenensis
L126	Rhodotorula dairenensis
L127	Rhodotorula dairenensis
L128	Rhodotorula dairenensis
L129	Rhodotorula dairenensis
L130	Trichosporon asahii
L131	Trichosporon asahii
L132	Trichosporon coremiiforme
L133	Trichosporon coremiiforme
L134	Trichosporon coremiiforme
L135	Trichosporon faecale
L136	Trichosporon faecale
L137	Trichosporon faecale
L138	Trichosporon loubieri
L139	Trichosporon loubieri
CCMA 896	Pichia kudriavzevii
CCMA 897	Pichia kudriavzevii
CCMA 898	Pichia kudriavzevii
CCMA 899	Pichia kudriavzevii
CCMA 900	Pichia kudriavzevii
CCMA 901	Pichia kudriavzevii
CCMA 902	Pichia kudriavzevii

Codes	Identification
CCMA 903	Pichia kudriavzevii
CCMA 904	Pichia kudriavzevii
CCMA 905	Pichia kudriavzevii
CCMA 906	Pichia kudriavzevii
CCMA 907	Pichia kudriavzevii
CCMA 908	Pichia kudriavzevii
CCMA 909	Pichia kudriavzevii
CCMA 910	Pichia kudriavzevii
CCMA 911	Pichia kudriavzevii
CCMA 912	Pichia kudriavzevii
CCMA 913	Pichia kudriavzevii
CCMA 914	Pichia kudriavzevii
CCMA 915	Pichia kudriavzevii
CCMA 916	Pichia kudriavzevii
CCMA 917	Pichia kudriavzevii
CCMA 918	Pichia kudriavzevii
CCMA 919	Pichia kudriavzevii
CCMA 920	Meyerozyma caribbica
CCMA 921	Meyerozyma caribbica
CCMA 922	Candida intermedia
CCMA 923	Candida ethanolica
CCMA 925	Pichia kudriavzevii
CCMA 926	Candida pararugosa
CCMA 927	Candida pararugosa
CCMA 928	Candida pararugosa
CCMA 929	Candida pararugosa
CCMA 930	Candida pararugosa
CCMA 931	Candida rugosa
CCMA 932	Candida rugosa
CCMA 933	Candida rugosa

Codes	Identification
CCMA 934	Kluyveromyces marxianus
CCMA 935	Pichia kudriavzevii
CCMA 936	Pichia kudriavzevii
CCMA 945	Rhodotorula dairenensis
CCMA 948	Pichia manshurica
CCMA 951	Pichia kudriavzevii
CCMA 952	Pichia kudriavzevii
CCMA 953	Candida rugosa
CCMA 954	Candida rugosa

Codes	Identification
CCMA 955	Candida rugosa
CCMA 956	Candida rugosa
CCMA 957	Candida rugosa
CCMA 958	Candida rugosa
CCMA 959	Candida rugosa
CCMA 960	Candida rugosa
CCMA 962	Candida ethanolica
CCMA 963	Pichia kudriavzevii

Table Supplementary 2 - Sample groups and population of isolated yeasts.

Animals	Total population[1]	Identification	Strains	Population by species [1]
A1 (Holstein)	4,84	*Candida pararugosa*	4	4,42
		Debaryomyces etchellsii	1	4,12
		Magnusiomyces capitatus	1	3,12
		Pichia kudriavzevii	3	3,9
		Pichia manshurica	3	3,6
		Rhodotorula dairenensis	1	4,12
A2 (Holstein)	4,99	*Candida ethanolica*	1	3,67
		Candida pararugosa	5	4,64
		Pichia kudriavzevii	3	4,2
		Pichia manshurica	3	4,15
A3 (Holstein)	4,9	*Candida pararugosa*	6	4,89
		Magnusiomyces capitatus	2	3,3
		Trichosporon faecale	1	3
A4 (Holstein)	4,91	*Candida pararugosa*	8	4,78
		Pichia manshurica	3	4,23
		Trichosporon coremiiforme	2	3,78
A5 (Holstein)	3,88	*Candida catenulata*	4	3,43
		Candida pararugosa	5	3,56
		Pichia kudriavzevii	4	3,12
A6 (Holstein)	3,9	*Candida catenulata*	3	3,37
		Candida pararugosa	4	3,3
		Candida rugosa	3	3,37
		Pichia kudriavzevii	6	3,12
A7 (Holstein)	3,79	*Candida catenulata*	1	2,57
		Candida ethanolica	2	2,3
		Candida pararugosa	6	3,62
		Candida rugosa	3	3,05
		Pichia kudriavzevii	1	2,52

Animals	Total population[1]	Identification	Strains	Population by species [1]
		Trichosporon asahii	1	2
		Trichosporon faecale	1	2
A8 (Holstein)	4,02	*Candida ethanolica*	5	3,57
		Candida pararugosa	1	3,08
		Candida rugosa	1	2,83
		Dipodascus ingens	2	2,6
		Pichia kudriavzevii	3	3,11
		Pichia manshurica	3	3,49
A9 (Holstein)	3,88	*Candida catenulata*	2	2,87
		Candida pararugosa	5	3,64
		Candida rugosa	1	3,58
		Pichia kudriavzevii	4	3,2
		Trichosporon asahii	1	2
		Trichosporon coremiiforme	1	2
		Trichosporon faecale	1	2
		Trichosporon loubieri	2	2,3
A10 (Nellore)	3,51	*Cryptococcus aspenensis*	1	2
		Cryptococcus flavus	1	2,7
		Cryptococcus liquefaciens	1	2
		Exophiala spinifera	1	2
		Kalmanozyma brasiliensis	5	3,4
A11 (Nellore)	3,6	*Cryptococcus aspenensis*	1	2,48
		Kalmanozyma brasiliensis	1	2,48
		Rhodotorula dairenensis	6	3,53

1 - Yeast population in log CFU/mL.

Table Supplementary 3 - Relative frequency (%) of yeast species identified from bovine fecal samples.

Identified Yeasts			Relative frequency (%)[1]										
			A1	**A2**	**A3**	**A4**	**A5**	**A6**	**A7**	**A8**	**A9**	**A10**	**A11**
Ascomycota	*Saccharomycetales*	*Ca. catenulata*	-	-	-	-	35.1	29.2	5.8	-	9.6	-	-
		Ca. rugosa	-	-	-	-	-	29.2	17.5	6.5	4.8	-	-
	Pichiaceae	*Ca. ethanolica*	-	5.9	-	-	-	-	3.1	36.1	-	-	-
		P. kudriavzevii	8.7	20.3	-	-	17.5	16.7	5.2	12.5	21.1	-	-
		P. manshurica	4.3	17.8	-	20.7	-	-	-	29.6	-	-	-
	Trichomonascaceae	*Ca. pararugosa*	57.0	55.9	96.3	72.0	47.4	25.0	65.3	11.5	57.9	-	-
	Debaryomycetaceae	*De. etchellsii*	14.2	-	-	-	-	-	-	-	-	-	-
	Dipodascaceae	*Di. ingens*	-	-	-	-	-	-	-	3.8	-	-	-
		M. capitatus	1.4	-	2.5	-	-	-	-	-	-	-	-
	Herpotrichiellaceae	*E. spinifera*	-	-	-	-	-	-	-	-	-	3.0	-
Basidiomycota	*Rhynchogastremataceae*	*Cr. aspenensis*	-	-	-	-	-	-	-	-	-	3.0	7.5
	Trimorphomycetaceae	*Cr. flavus*	-	-	-	-	-	-	-	-	-	15.2	-
	Filobasidiaceae	*Cr. liquefaciens*	-	-	-	-	-	-	-	-	-	3.0	-
	Ustilaginaceae	*K. brasiliensis*	-	-	-	-	-	-	-	-	-	75.8	7.5
	Sporidiobolaceae	*R. dairenensis*	14.2	-	-	-	-	-	-	-	-	-	85.0
	Trichosporonaceae	*T. asahii*	-	-	-	-	-	-	1.6	-	1.3	-	-
		T. coremiiforme	-	-	-	7.3	-	-	-	-	1.3	-	-
		T. faecale	-	-	1.3	-	-	-	1.6	-	1.3	-	-
		T. loubieri	-	-	-	-	-	-	-	-	2.6	-	-

1 – Relative frequency (%) calculated considering the population of each identified yeast species in relation to the total population of yeasts in the analyzed samples.