EMERSON JOSUE MARTINEZ JIMENEZ

FERMENTAÇÃO ANAERÓBICA AUTOINDUZIDA (SIAF) COM E SEM INOCULAÇÃO DE LEVEDURAS: VIABILIDADE DA SEMENTE, COMPOSIÇÃO QUÍMICA E QUALIDADE SENSORIAL DO CAFÉ

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-graduação em Ciência dos Alimentos, área de concentração em Ciência dos Alimentos, para obtenção do título de Doutor.

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SELF-INDUCED ANAEROBIOSIS FERMENTATION (SIAF) WITH AND WITHOUT YEAST INOCULATION: SEED VIABILITY, CHEMICAL COMPOSITION AND COFFEE SENSORIAL QUALITY

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A Deus, por sempre estar comigo em toda minha caminhada

Dedico

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RESUMO GERAL

A fermentação do café ocorre devido a produção de metabolitos produzidos por microrganismos epifíticos presentes no próprio fruto e são transferidos ´para o interior do grão. Além disso, durante a fermentação, processos metabólicos de germinação são iniciados dentro da semente, afetando a viabilidade do embrião, a composição química e a qualidade sensorial do café. Nesse contexto, nosso objetivo foi avaliar os efeitos da fermentação do café na viabilidade das sementes e na qualidade sensorial da bebida utilizando leveduras como culturas iniciadoras (*Saccharomyces cerevisiae* CCMA 0543, *Candida parapsilosis* CCMA 0544 e *Torulospora delbrueckii* CCMA 0684), por fermentação anaeróbica induzida (SIAF) em *Coffea arabica* L. variedade Topazio amarelo, por processamento natural e despolpado. Monitoramento das leveduras foi realizado por reação em cadeia da polimerase em tempo real (qPCR), análise de ácidos orgânicos (cítrico, málico, succínico e lático) e açúcares (sacarose, glicose e frutose) por cromatografia líquida de alta eficiência (HPLC). Compostos voláteis por cromatografia gasosa-espectrometria de massa (GC-MS). A viabilidade e os danos das sementes foram monitorados por teste de tetrazólio, condutividade elétrica, teste de germinação de sementes e análise enzimática por eletroforese em gel (catalase, álcool desidrogenase, esterase e isocitrato liase). As leveduras *S. cerevisiae* e *T. delbrueckii* apresentaram as maiores populações durante a fermentação em comparação com o tratamento de controle SIAF e o processo convencional sem inoculação. Observou-se diminuição na concentração de açúcares (sacarose, glicose e frutose) e ácidos (cítrico, málico e succínico). Os tratamentos processados pelo SIAF apresentaram produção de ácido lático no final da fermentação (o tratamento controle SIAF apresentou a maior produção com 8,33 g/kg no café descascado e 3,95 g/kg no café natural). Enzimas chave dos processos metabólicos de germinação (isocitrato liase e endo-β-mananase) demonstraram o início da germinação durante a fermentação do café, bem como processos de defesa contra estresse oxidativo (esterase e catalase) e ambiental (anaerobiose determinado por álcool desidrogenase). Alterações na qualidade fisiológica das sementes foram identificadas, incluindo diminuição da viabilidade embrionária pelo teste de tetrazólio (o tratamento inoculado com *S. cerevisiae* foi o tratamento com menor % de viabilidade embrionária, com 52,5 no café natural e 60,0 no café despolpado). O tratamento convencional processado com café natural não apresentou diminuição na % de germinação das sementes. Todos os cafés obtiveram notas superiores a 80 pontos. Os cafés com maior pontuação sensorial foram obtidos de inoculados e fermentados pelo SIAF; levedura *T. delbrueckii* (86,50) em café despolpado e levedura *C. parapsilosis* (85,90) em café natural. Os cafés fermentados pelo SIAF foram caracterizados pelos aromas e sabores cítricos, caramelo, mel, chocolate e castanha. O processo de fermentação do café por SIAF com inoculação de levedura afetou a viabilidade do grão de café, mas não a qualidade sensorial da bebida, indicando que a utilização de starters de levedura pelo método SIAF favorece a produção de cafés especiais com características sensoriais diferenciadas.

Palavras-chave: Inoculação. Leveduras. Cafés especiais. Fisiologia de Sementes. Qualidade sensorial. SIAF.

GENERAL ABSTRACT

Coffee fermentation occurs due to the production of metabolites produced by epiphytic microorganisms present in the fruit itself and transferred to the interior of the grain. In addition, during fermentation, germination metabolic processes are initiated within the seed, affecting the embryo's viability, the chemical composition, and the sensory quality of the coffee. In this context, our objective was to evaluate the effects of coffee fermentation on seed viability and sensory quality of the beverage using yeast as starter cultures (*Saccharomyces cerevisiae* CCMA 0543, *Candida parapsilosis* CCMA 0544 and *Torulospora delbrueckii* CCMA 0684), by induced anaerobic fermentation (SIAF) in *Coffea arabica* L. Variety Yellow Topaz, by natural and pulped processing. Yeast monitoring was performed by real-time polymerase chain reaction (qPCR), analysis of organic acids (citric, malic, succinic, and lactic) and sugars (sucrose, glucose, and fructose) by high-performance liquid chromatography (HPLC). Volatile compounds by gas chromatography-mass spectrometry (GC-MS). Seed viability and damage were monitored by tetrazolium test, electrical conductivity, seed germination test, and enzymatic analysis by gel electrophoresis (catalase, alcohol dehydrogenase, esterase, and isocitrate lyase). Yeasts *S. cerevisiae* and *T. delbrueckii* showed the highest populations during fermentation compared with the control SIAF treatment and the conventional process without inoculation. A decrease in the concentration of sugars (sucrose, glucose, and fructose) and acids (citric, malic, and succinic) was observed. Treatments processed by SIAF showed lactic acid production during the end of fermentation (the control treatment SIAF showed the highest production with 8.33 g/kg in pulped coffee and 3.95 g/kg in natural coffee). Key enzymes of the metabolic processes of germination (isocitrate lyase and e endo-β-mannanase) demonstrated the beginning of germination during coffee fermentation, as well as processes of defense against oxidative (esterase and catalase) and environmental stress (anaerobiosis determined by alcohol dehydrogenase). Changes in seed physiological quality were identified, including decreased embryonic viability by tetrazolium test (the treatment inoculated with *S. cerevisiae* was the treatment with the lowest % embryonic viability, with 52.5 in natural coffee and 60.0 in pulped coffee). The conventional treatment processed by natural coffee did not show a decrease in the % of seed germination. All coffees obtained scores greater than 80 points. The coffees with the highest sensory score were obtained from inoculated and fermented by SIAF; yeast *T. delbrueckii* (86.50) in pulped coffee and yeast *C. parapsilosis* (85.90) in natural coffee. Coffees fermented by SIAF were characterized by aromas and flavors of citrus, caramel, honey, chocolate, and chestnut. The coffee fermentation process by SIAF with yeast inoculation affected the viability of the coffee bean but not the sensory quality of the drink, indicating that the use of yeast starters by the SIAF method favors the production of special coffees with differentiated sensory characteristics.

Keywords: Inoculation. Yeasts. Specialty coffees. Seed Physiology. Sensory quality. SIAF.

SUMÁRIO

PRIMEIRA PARTE

1 INTRODUÇÃO

O Brasil é o maior produtor e exportador de café no mundo, na safra 2021/22, produziu 60.4 milhões de sacas de café, seguido pelo Colômbia, Etiópia, Honduras e Peru (OIC, 2023). Além da produção os consumidores estão constantemente em busca de cafés de boa qualidade, devido ao grande crescimento e disponibilidade dos cafés especiais o qual gera uma alta demanda dos consumidores (CÓRDOBA *et al*., 2021). Além disso a qualidade do café é definida por fatores intrínsecos e extrínsecos como: genética da planta, estado de maturação do fruto, condições ambientais, práticas agrícolas, clima (temperatura, umidade, precipitação e vento), altitude, disponibilidade de luz, técnicas de pós-colheita, (ABREU *et al*., 2017; HAILE; KANG, 2019).

Desta forma o processamento pós-colheita é um dos fatores onde o produtor pode influenciar a qualidade do café, dependendo do método de processamento: seco, semisseco e úmido, os quais utilizam diferentes técnicas e tecnologias com e sem utilização de água, para a retirada da casca e mucilagem do café, obtendo dessa forma o grão de café (BOREM, 2008; BRANDO; BRANDO, 2015). Assim durante a pós-colheita é desenvolvida uma fermentação no café caracterizada pela presença de diferentes grupos microbianos tais como bactérias, fungos e leveduras. Dessa maneira estes microrganismos através da fermentação consumem os nutrientes disponíveis, produzindo metabólitos e enzimas que influenciam na qualidade do café, através do intercambio de compostos do exterior para o interior do grão do café (HADJ SALEM *et al*., 2020; SCHWAN *et al*., 2022; SILVA *et al*., 2013).

Nos últimos anos, o processamento de fermentação de café tem se concentrado em alterar a composição química do café e desenvolver diferentes perfis sensoriais através da utilização de novas tecnologias como a Fermentação Anaeróbica Autoinduzida (SIAF) pelo metabolismo microbiano (BRAGA *et al*., 2023; JIMENEZ *et al*., 2023; PEREIRA *et al*., 2022). Este método consiste na inoculação de microrganismos (leveduras e bactérias) em biorreatores fechados provocando anaerobioses pela produção gradual de CO_2 impulsionada pelo metabolismo microbiano (PEREIRA *et al*., 2022). Essa tecnologia melhora o desempenho fermentativo de bactérias láticas e leveduras, aumentando a produção de metabólitos e a qualidade sensorial da bebida (CASSIMIRO *et al*., 2022; DA MOTA *et al*., 2022).

No entanto, durante a fermentação e secagem do café, processos fisiológicos do metabolismo germinativo são desenvolvidos no interior da semente. Através de enzimas chaves

como a isocitrato liasse, catalase, esterase, que indicam o metabolismo ativo da semente (NUNES DE FREITAS *et al*., 2017; SELMAR *et al*., 2006; TAVEIRA *et al*., 2015). Este processo germinativo é evidentemente reduzido pela falta de condições adequadas (oxigênio, temperatura, atividade da água, ruptura das membranas celulares entre outras) provocando a dormência na semente, diminuição das reservas de nutrientes, assim como diminuição da viabilidade da semente com um possível impacto na qualidade do café (JIMENEZ *et al*., 2023; SELMAR; KLEINWÄCHTER; BYTOF, 2015; TAIZ *et al*., 2017a).

Neste metabolismo ativo de germinação das sementes são desenvolvidas alterações na integridade das membranas celulares do embrião, a germinação, teor de ácidos orgânicos e carboidratos, impactando na qualidade do café através da migração de compostos no interior do grão (KLEINWÄCHTER; SELMAR, 2010; SELMAR *et al*., 2006; TAVEIRA *et al*., 2015).

Na atualidade a fermentação do café utiliza culturas iniciadoras para controlar o processo fermentativo e assim obter cafés com melhorias na composição química e sensorial (DA MOTA *et al*., 2020; SCHWAN *et al*., 2022). Nesse contexto as leveduras *Saccharomyces cerevisiae* (CCMA 0543), *Candida parapsilosis* (CCMA 0544), e *Torulospora delbrueckii* (CCMA 0684), foram estudadas como potenciais culturas iniciadoras (EVANGELISTA *et al*., 2014; MARTINEZ *et al*., 2019; RIBEIRO *et al*., 2017). Estas leveduras isoladas e identificadas nos processos de fermentação do café (*Coffea arabica* L.) foram testadas em diferentes métodos de processamento do café, mostrando a capacidade de produzir enzimas que degradam a mucilagem (atividade pectinolítica), inibem o crescimento de fungos produtores de micotoxinas e produzem componentes ativos de sabor (DA MOTA *et al*., 2020; DE SOUZA *et al*., 2017).

Desta maneira, o objetivo deste trabalho foi avaliar os efeitos da fermentação do café na viabilidade das sementes, composição química e na qualidade sensorial da bebida utilizando como culturas iniciadoras, as leveduras: *Saccharomyces cerevisiae* (CCMA 0543), *Candida parapsilosis* (CCMA 0544), e *Torulospora delbrueckii* (CCMA 0684), através de fermentação por anaerobioses induzida (SIAF) em *Coffea arabica* L. variedade Topazio amarelo, por processamento natural e despolpado.

2 REFERENCIAL TEÓRICO

2.1 Generalidade do café

O café é uma das bebidas mais populares do mundo, com cerca de quatrocentos bilhões de xícaras sendo consumidas todos os anos (SPENCE; CARVALHO, 2020). É uma cultura

comercial de importância econômica primária em países produtores como Brasil, Colômbia, Vietnã, Etiópia e Indonésia (CONAB, 2019). O café é pertencente ao gênero *Coffea* à família Rubiaceae e compreende 103 – 124 espécies dependendo da amplitude da classificação taxonômica (DAVIS *et al*., 2011).

Entretanto, apenas duas espécies são responsáveis por contabilizar toda a produção global de café; *Coffea arabica* L., muitas vezes referido como 'Arabica', responsável por aproximadamente o 60% da produção mundial e *Coffea canephora* Pierre ('Robusta') responsável pelos 40% restantes (CHENG *et al*., 2016; HALL; TREVISAN; DE VOS, 2022). A espécie do gênero *Coffea,* variedade *C. arabica* L., é considerada um café com um perfil sensorial mais apreciado a nível internacional, descrito como aromático, saboroso e agradável ao paladar em comparação com *C. canephora* Pierre considerado mais amargo e com perfil menos aromático. No entanto, as preferências de gosto (locais, nacionais, culturais) devem ser consideradas, pois diferem consideravelmente (SENINDE; CHAMBERS, 2020).

2.2 Qualidade do café

A qualidade do café é um conjunto de caraterísticas sensoriais do grão ou da bebida que caracteriza os sabores e gostos do café, sem excluir os atributos físicos, químicos, sensoriais e higiênico sanitários, que proporcionam prazer e segurança ao consumidor. Além disso, a qualidade do café pode ser considerada como a bebida que apresenta sabor e aromas agradáveis, acidez natural e suavidade ao paladar, assim como efeitos positivos na saúde e no estado de alerta, como origem geográfica e aspectos ambientais e sociais, além das condições de preços, livre de defeitos e de acordo com as normas higiênico sanitárias (BOREM, 2008; HAILE; KANG, 2019).

Esta qualidade pode ser influenciada por alguns fatores: físicos (tamanho, cor, uniformidade e grãos com defeito), fatores químicos (componentes-chave como cafeína, trigonelina, sacarose e ácidos clorogênicos, considerados significativos por influenciar a qualidade do café) e fatores sensoriais (sabor, ou seja, a qualidade da xícara) (BORÉM *et al*., 2019; CHENG *et al*., 2016; MARQUES *et al*., 2008). No entanto, todos esses fatores são englobados pelas considerações gerais de genética e melhoramento de plantas, que dependem da constituição genética ou genótipo, das condições ambientais para a qual o genótipo está submetido e a interação entre eles (FIGUEIREDO *et al*., 2015).

Além disso hoje em dia a qualidade de café é medida através da análise sensorial por juízes treinados pelo método de avaliação sensorial da Specialty Coffee Association (SCA),

baseado em uma análise descritiva quantitativa da bebida, avaliando os atributos: fragrância, aroma, sabor, retrogosto, acidez, corpo, uniformidade, equilíbrio, doçura, limpeza, defeitos, e pontuação geral, a qual consiste em dar uma pontuação geral de 0-100, classificando aqueles cafés com pontuações acima de 80 como cafés especiais (LINGLE, 2011; SCA, 2018). Os cafés especiais, são cafés da mais alta qualidade produzidos em quantidades limitadas e rastreáveis de uma única origem (terroir). Eles são caracterizados por um sabor e aroma distintos e superiores, o que é resultado das condições específicas de cultivo e colheita (JESZKA, 2022). Desse modo para os consumidos, o sabor é sem dúvida o aspecto mais importante de um bom café (SUNARHARUM; WILLIAMS; SMYTH, 2014).

2.3 Processamento pós-colheita do café

Antigamente o principal objetivo do processamento do café era a remoção da camada de mucilagem para facilitar a secagem do grão (BELITZ; GROSCH; SCHIEBERLE, 2009). Na atualidade o processamento pós-colheita visa a remoção da polpa dos frutos maduros, sendo um passo fundamental para a obtenção de alta qualidade no produto (CORTÉS-MACÍAS *et al*., 2022; HAMEED; HUSSAIN; SULERIA, 2020). Assim, os frutos do café são transformados em grãos de café beneficiados com porcentagens de umidade em torno de 11% a 12% a partir da retirada das partes que envolvem os grãos (HAMEED; HUSSAIN; SULERIA, 2020). Além disso, deve-se evitar fermentações indesejáveis e deterioração dos frutos que possam influenciar negativamente a qualidade sensorial do café (BARRIOS-RODRÍGUEZ *et al*., 2021).

Assim o processamento de café é possível através de três tipos de métodos: seco, semisseco e úmido (BRANDO & BRANDO, 2015) (Figura 1).

a) Método cereja natural: a fruta inteira recém-colhida é fermentada e seca em plataformas, após as quais os grãos de café são removidos por beneficiamento (removida a camada de casca que cobre os grãos de café secos) (SILVA *et al*., 2000).

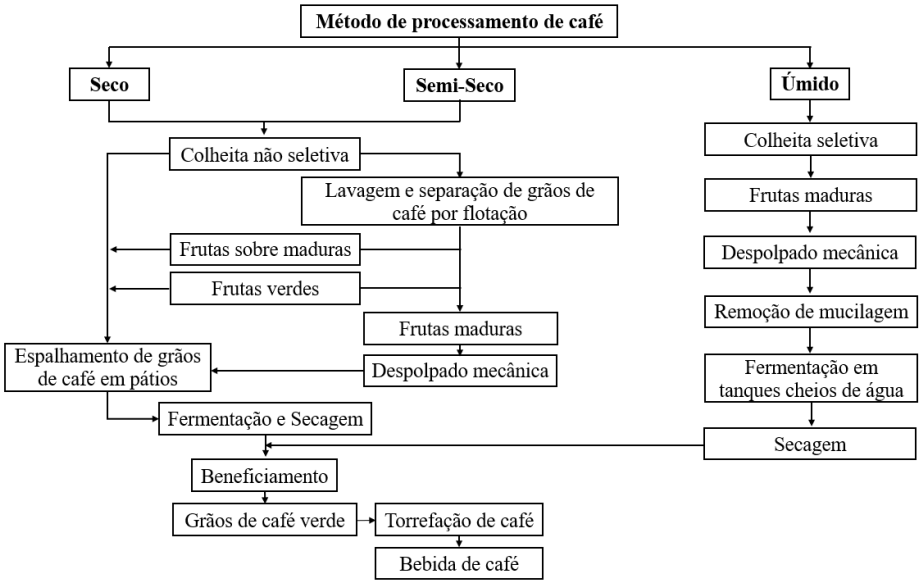
b) Método cereja descascado: a casca de café, a polpa e parte ou toda a mucilagem são removidas mecanicamente e, em seguida, o café é fermentado e seco (BRANDO; BRANDO, 2015). É considerado um processamento intermediário entre o processamento seco e o úmido e que consome mais água do que o processamento natural direto. A cereja do café é então seca ao sol, com grande parte da mucilagem ainda presa, em uma cama de secagem elevada ou em um pátio (SILVA, 2015).

c) Método úmido: a casca e a polpa são removidas mecanicamente, deixando a

silagem aderida ao fruto. Esses cafés despolpados são então transferidos para tanques de água, onde são permitidos fermentar por 6 a 72 h (dependendo da temperatura ambiente), durante os quais a mucilagem restante é degradada e solubilizada. Em seguida, os grãos são removidos dos tanques e secos ao sol (SILVA, 2015). Do mesmo modo o método úmido, é um processo de pós-colheita usado para manter a suavidade do café arábica, envolve a remoção mecânica da casca da fruta (exocarpo ou polpa) para expor a camada de geleia (mesocarpo ou mucilagem) aderida à superfície do grão. No processo de degradação da mucilagem, os microrganismos no ambiente utilizam a mucilagem como substrato para seus processos metabólicos, gerando ácidos orgânicos, entre outros (PEÑUELA-MARTÍNEZ; ZAPATA-ZAPATA; DURANGO-RESTREPO, 2018; SUNARHARUM; WILLIAMS; SMYTH, 2014).

Durante este processamento pós-colheita surgem mudanças na composição química dos grãos de café verde podendo ser atribuídas aos processos metabólicos, que são específicos para cada tipo de tratamento pós-colheita (BRESSANI *et al*., 2021; SELMAR *et al*., 2006). Provavelmente pela variação de fatores como a remoção de algumas partes que constituem o fruto (casca, mucilagem), que contêm nutrientes (açúcares, aminoácidos, proteínas) assim como hormônios e enzimas presentes que favorecem a germinação do embrião (MUNYENDO *et al*., 2021; RIBEIRO *et al*., 2017). Além disso, em todos esses métodos de processamento, é realizada uma fermentação espontânea por microrganismos epifíticos do café que elimina qualquer mucilagem ainda aderida no fruto que ajuda a melhorar o sabor da bebida pelos metabólitos microbianos (SCHWAN *et al*., 2022; SILVA, 2015; WANG; WU; SHYU, 2014).

Figura 1 Métodos de processamento pós-colheita de café.



Fonte: Adaptado de BRANDO & BRANDO (2015).

Esta fermentação ocorre através de diferentes interações dos microrganismos; (i) microrganismos e substrato; (ii) microrganismos e metabólitos; e (iii) diferentes grupos microbiológicos em nível de espécie e cepa, induzindo alterações bioquímicas e/ou físicas no substrato que também influenciam a fermentação (NIELSEN; ARNEBORG; JESPERSEN, 2015). Além do desenvolvimento de processos catabólicos de oxidação de substâncias orgânicas, principalmente açúcares que são transformados em energia e em compostos mais simples, como etanol, ácido lático, ácido acético e ácido butírico (PUERTA QUINTERO; MEJÍA; OSORIO BETANCOUR, 2012). Assim é possível que esses metabolitos microbianos produzidos durante a fermentação, como as enzimas presentes migram ao interior do grão provocando mudanças na composição química e sensorial do café (HADJ SALEM *et al*., 2020; HAILE; KANG, 2019; PEREIRA *et al*., 2022; WANG *et al*., 2020).

2.3.1 Influência do método de processamento

O processamento natural ou seco é a forma mais antiga, barata e fácil de transformar os frutos maduros de café em grãos de café beneficiados. Onde os frutos de café são secos ao sol (fermentação), produzindo um café com forte aroma, acidez moderada, corpo intenso e agradável (HAMEED; HUSSAIN; SULERIA, 2020; SCHOLZ *et al*., 2019). O processamento natural é a maneira mais difícil de proteger e manter a alta qualidade do café, pois os frutos inteiros são secos ao sol ou à máquina sob luz solar direta em superfícies planas, canteiros elevados (terra nua, piso cimentado ou tijolo, mesas com tela de arame, esteiras de bambu, asfalto ou superfícies de madeira) ou em máquinas de secagem junto com as camadas externas intactas do fruto fornecendo uma bebida com corpo pesado e atributos doces, suaves e complexos (HAMEED; HUSSAIN; SULERIA, 2020; SANZ-URIBE *et al*., 2017). Também há um maior número de grãos defeituosos com defeitos de café terroso, mofado e esverdeado que são encontrados no café processado a seco (TADESSE; JEMAL; ABEBE, 2015).

Pesquisas comparando tratamentos usando os mesmos materiais iniciais e realizadas especificamente para testar os diferentes métodos de processamento demonstraram que o próprio método de processamento afeta a química do grão e a qualidade subsequente (HALL; TREVISAN; DE VOS, 2022). Por exemplo, os grãos obtidos pelo método de processamento a seco superaram os do método de processamento por via úmida em certas características bioquímicas, podendo ser correlacionadas com a qualidade. Grãos processados por via seca apresentam níveis mais altos de sacarose, teores mais baixos de trigonelina e 3-CQA em comparação com grãos processados por via úmida (DIEGO *et al*., 2016; TOLESSA *et al*.,

2019).

De igual forma Scholz *et al*. (2019) compararam a composição química de cafés processados pelos processamentos descascados e naturais, observando maiores teores de lipídios, cafeína, compostos fenólicos, ácidos clorogênicos e acidez nos cafés processados pelo método natural em comparação com o processamento descascado. Desta forma observaram a influência do método de processamento na composição química do café, a qual pode ser influenciada pelo método de processamento elegido. Finalmente (HAMEED; HUSSAIN; SULERIA, 2020), apresentam vantagens e desvantagens dos processamentos seco, semisseco e úmido na Tabela 1.

Tabela 1 Vantagens e desvantagens dos métodos de processamento do café.

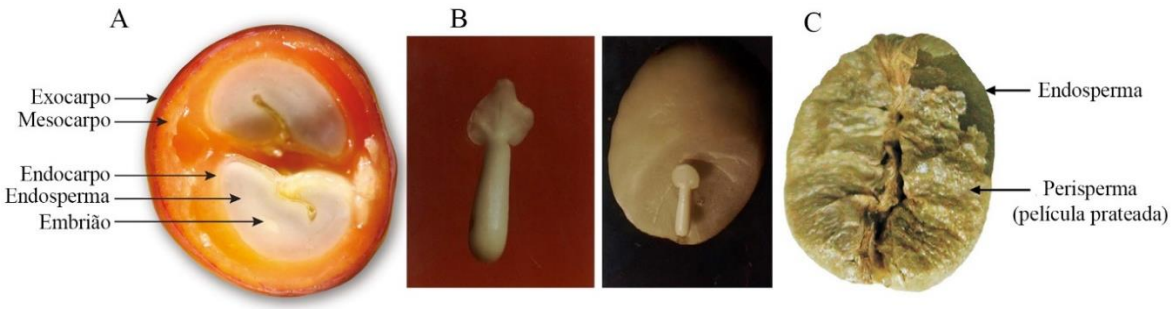
Métodos de processamento	Vantagens	Desvantagens
Seco	Processo simples	Processo demorado (3-4 semanas)
	Baixo custo	Sobre fermentação
	Fácil de executar e manusear	Contaminação por fungos/mofo
	Menos trabalhoso	Produção de ocratoxina A
	Maior taxa de secagem	Maior taxa de grãos defeituosos
	Cerejas maduras e imaturas podem ser usadas	Menos mercado de exportação devido às chances de matéria estranha
	Nível mais alto de proteínas (de armazenamento) e hexoses (glicose e frutose) em infusão	Atributos de qualidade menos desejáveis (corpo pesado com doçura e atributos complexos)
	Amigo do ambiente	
Úmido	Economia de tempo	Caro
	Fermentação controlada	Trabalhoso
	Aumentar o conteúdo de estaquiose e sorbitol	Precisa de arranjos adicionais
	Maior teor de CGA e trigonelina	Cerejas maduras são necessárias
	Maior produção de ácido orgânico	
	Mercado de exportação atraente	Menos amigo do ambiente
	Menor taxa de grãos defeituosos	
	Menos chances de produção de OTA	
Semi seco	Transição do sistema seco e semiúmido	Caro
	Cerejas pouco maduras, maduras podem se usar	Menos praticado globalmente
	Maior teor de cafeína	Trabalhoso
	Nível intermediário de CGA	Menos amigo do ambiente
	Conteúdo de trigonelina mais baixo	
	Sabor mais limpo com menos acidez e corpo	

Fonte: (HAMEED; HUSSAIN; SULERIA, 2020).

2.4 Anatomia e composição química do café

Segundo Borém (2008) o conhecimento da anatomia e da composição química do fruto e da semente do cafeeiro é fundamental para a adequada compreensão dos fenômenos fisiológicos, físicos e químicos que ocorrem durante a pós-colheita do café. Esta composição química do café verde e a qualidade final do café podem ser alteradas pelo tipo de tratamento pós-colheita (ELHALIS; COX; ZHAO, 2023; SANZ-URIBE *et al*., 2017). Desta forma cabe mencionar que o fruto de café é constituído por seis partes: o exocarpo (casca), mesocarpo (mucilagem), endocarpo (pergaminho), e a semente composta pelo perisperma (película prateada), endosperma e embrião (Figura 2) (BOREM, 2008; FERREIRA *et al*., 2019).

Figura 2 Morfologia da semente de café**.**



A. Corte transversal de um fruto de café. **B.** Embrião de *C. arabica* L., (esquerda) isolado e (direita) com a superfície externa do endosperma cortada para expor o embrião. **C.** Perisperma (película prateada).
Fonte: (FERREIRA *et al*., 2019).

Além disso, é importante mencionar que a camada mucilaginosa dos grãos de café está composta por 84,2% de água, 8,9% de proteína, 4,1% de açúcar, 0,91% de substâncias pécticas e 0,7% de cinza (BELITZ; GROSCH; SCHIEBERLE, 2009). Também é fornecida uma análise das diferenças na composição química entre o café arábica e o café robusta é fornecida na Tabela 2.

Tabela 2 Composição química do café verde (variedades arábica e robusta) *a, b. (Continua).

Componentes	Arábica	Robusta	Constituinte
Carboidratos solúveis	9-12.5	6-11.5	
Monossacarídeos	0.2-0.5		Frutose, glicose, galactose, arabinose, (traços).
Oligossacarídeos	6-9	3-7	Sacarose (> 90%), rafinose (0-0,9%), estaquiose (0-0,13%).

Tabela 2 Composição química do café verde (variedades arábica e robusta) *a, b. (Conclusão).

Componentes	Arábica	Robusta	Constituinte
Polissacarídeos	3-4		Polímeros de galactose (55-65%), manose (10-20%), arabinose (20-35%), glicose (0-2%).
Polissacarídeos insolúveis	46-53	34-44	
Hemiceluloses	5-10	3-4	Polímeros de galactose (65-75%), arabinose (25-30%). manose (0-10%).
Celulose β(I-4) mannan	41-43	32-40	
Ácidos e fenóis			
Ácidos voláteis	0.1		
Ácidos alifáticos não voláteis	2-2.9	1.3-2.2	Ácido cítrico, ácido málico, ácido quínico
Ácido clorogênico	6.7-9.2	7.1-12.1	Ácido mono-, dicafeoil- e feroililquinico
Lignina	1-3		
Lipídios	15-18	8-12	
Cera	0.2-0.3		
Óleo	7.7-17.7		Principais ácidos graxos: 16: 0 e 18: 2 (9,12).
Compostos N	11-15		
Aminoácidos livres	0.2-0.8		Principais aminoácidos: Glu. Asp. Asp-NH2.
Proteínas	8.5-12		
Cafeína	0.8-1.4	1.7-4.0	Traços de teobromina e teofilina.
Trigonelina	0.6-1.2	0.3-0.9	
Minerais	3-5.4		

*a Valores em % de sólidos *b Teor de água do café cru: 7-13% *c Componentes principais: ácido 5-cafeoilquínico (ácido clorogênico: Arábica 3,0–5,6%; Robusta 4,4–6,6%).

Fonte: (BELITZ; GROSCH; SCHIEBERLE, 2009)

2.5 Influência do processamento na química do café

A composição química dos grãos de café verde é muito complexa, incluindo mais de 1.000 substâncias com diferentes propriedades químicas e físicas, estas sustâncias são divididas em dois grupos de compostos, sendo eles os voláteis e não voláteis (DE PEÑA; LUDWIG; CID, 2019).

Os principais precursores de aroma são carboidratos insolúveis (celulose e hemicelulose), carboidratos solúveis (arabinose, frutose, galactose, glicose, sacarose, rafinose) (FADAI *et al*., 2017; POISSON *et al*., 2018; WANG *et al*., 2020), ácidos orgânicos (cítrico, málico, oxálico, tartárico, pirúvico, quínico, clorogênico, acético e outros), lipídios formados por ácidos graxos (tocoferóis, esteróis, ésteres de ácidos graxos, diterpenos e outros), aminoácidos, proteínas voláteis (DE PEÑA; LUDWIG; CID, 2019), e compostos voláteis como: hidrocarbonetos, álcoois, aldeídos, cetonas, ácidos carboxílicos, ésteres, pirazinas,

pirroles, piridinas, outras bases, compostos enxofres, furanos, fenóis, oxazoles e outros (DE PEÑA; LUDWIG; CID, 2019; FLAMET, 2002).

No entanto, durante o processamento de pós-colheita (fermentação) são geradas alterações físico-químicas nos grãos (alterações nos compostos voláteis e não voláteis), gerando sabores e aromas adicionais através dos metabólitos produzidos neste devido processo (EVANGELISTA *et al*., 2014; JOËT *et al*., 2010; KNOPP; BYTOF; SELMAR, 2006; SILVA *et al*., 2013). Esses metabólitos estão relacionados à produção microbiana de metabólitos finais, incluindo ésteres, ácidos orgânicos, aldeídos e álcoois superiores, que se difundem nos grãos e fornecem atributos sensoriais únicos (ELHALIS *et al*., 2020; ELHALIS; COX; ZHAO, 2020; FELDMANN, 2012).

2.5.1 Compostos voláteis e não voláteis no café

A avaliação dos compostos voláteis e não voláteis do café afeta diretamente sua qualidade sensorial, sendo de extrema importância para definir a qualidade final do produto. A presença desses compostos bem como sua concentração são influenciados pelos microrganismos (leveduras e bactérias láticas principalmente) presente durante o processo fermentativo. Diferentes ácidos orgânicos já foram detectados como lático, cítrico, málico, succínico, acético e tartárico. Esses ácidos contribuem para a acidez da bebida, sendo um atributo importante para a qualidade do café em combinação com a doçura, amargor e aroma. Entretanto, ácidos indesejados como butírico e propiônico conferem à bebida sabores estranhos como cebola e a sua presença mostra que os grãos foram fermentados excessivamente (BRESSANI *et al*., 2020; SUNARHARUM; WILLIAMS; SMYTH, 2014).

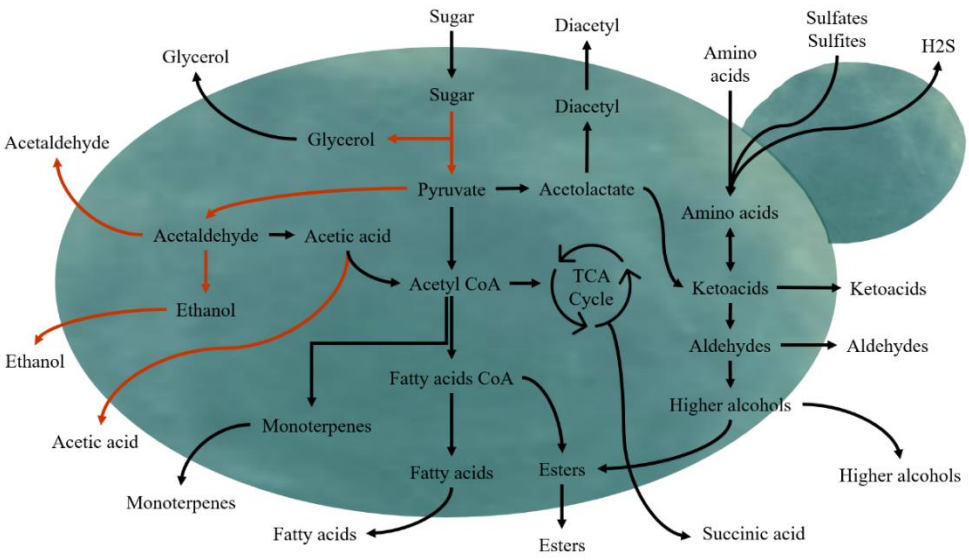
Martinez *et al*. (2019) avaliaram o efeito de culturas iniciadoras bacterianas e de leveduras na formação de ácidos orgânicos durante a fermentação úmida de cafés arábica, identificando oito ácidos orgânicos. O controle não apresentou ácido málico, succínico, lático e acético. Os ácidos málico, lático e acético só foram detectados em tratamentos com bactérias e estes podem estar relacionados à sua atividade metabólica. Já o ácido succínico foi detectado em todos as culturas bacterianas e em duas leveduras, *Candida parapsilosis* CCMA 0544 e *Torulaspora delbrueckii* CCMA 0684. O ácido succínico é um dos principais ácidos orgânicos produzido por leveduras e é formado no ciclo do glioxilato por oxidação do isocitrato, bem como no ciclo redutor do ácido cítrico.

2.6 Leveduras e seu comportamento durante a fermentação do café

As leveduras, possuem grande influência através da geração de diferentes compostos que influenciam o aroma por meio do metabolismo de carbono e nitrogênio. Além disso, cepas de leveduras isoladas do café são capazes de promover a degradação da pectina por meio da produção de diferentes enzimas hidrolíticas como poligalacturonase, pectina liase e pectina metilesterase. A hidrólise da pectina libera açúcar simples como fonte de carbono adicional para o metabolismo da levedura e formação de aroma (SILVA *et al*., 2013).

Etanol, acetaldeído e ácido acético são os metabólitos primários produzidos pela levedura durante a fermentação do café. Entretanto, junto com etanol, muitos compostos de sabor de baixo peso molecular são produzidos durante o processo de remoção da mucilagem, como ésteres, álcoois superiores, aldeídos, cetonas e terpenos (DEAK, 2008; FELDMANN, 2012; PRETORIUS, 2000). Dependendo do tipo de levedura e das condições ambientais (presença ou ausência de oxigênio, temperatura, a_w, pH), o crescimento de leveduras pode ser favorecido ou limitado permitindo o crescimento ou inibição de outros microrganismos presentes no café (bactérias ácido-láticas, mesófilos e fungos) provocando diferentes vias metabólicas (Figura 3) neste processo (EVANGELISTA *et al*., 2015; MARTINS *et al*., 2019; PEREIRA *et al*., 2022). Além disso, são observadas interações de microrganismos, com as leveduras que ajudam a criar as condições ideais para seu crescimento (como a redução do pH e a_w) (DEAK, 2008; FELDMANN, 2012; PRETORIUS, 2000).

Figura 3 Potenciais compostos de aroma que podem ser gerados pelas leveduras durante a fermentação do café a partir dos precursores presente no fruto.

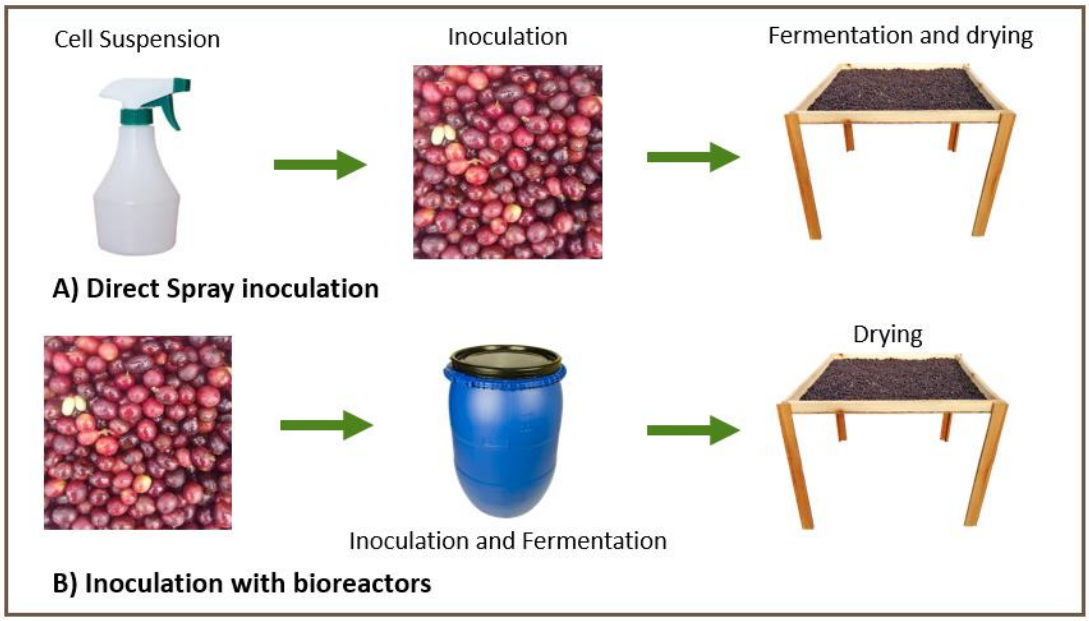


Fonte: Adaptado de (DEAK, 2008; FELDMANN, 2012; PRETORIUS, 2000).

2.7 Fermentação Anaeróbica Autoinduzida (SIAF)

Cassimiro *et al*. (2023) definem a fermentação anaeróbica autoinduzida (SIAF) como um método de fermentação em que a condição anaeróbia é gradualmente formada pelo metabolismo microbiano que utiliza o O_2 restante para suas reações metabólicas, liberando CO_2, compostos voláteis e não voláteis. Este método tem sido considerado para substituir ou complementar as comunidades microbianas naturais tradicionalmente utilizadas (epifíticas), com o objetivo específico de direcionar o processo de fermentação de forma deliberada, torná-lo mais uniforme e o resultado mais previsível, a fim de produzir grãos de café com melhor qualidade sensorial (BRESSANI *et al*., 2021; HAILE; KANG, 2019; WANG *et al*., 2020). Além disso, o método SIAF impacta positivamente o desempenho fermentativo de LAB e leveduras durante o processamento de café (DA MOTA *et al*., 2020; JIMENEZ *et al*., 2023; PEREIRA *et al*., 2022).

Figura 4 Processamento do café natural com diferentes formas de inoculação.



A) inoculação direta da levedura no fruto e levada para terreiro suspenso; B) inoculação da levedura em biorreator (SIAF), fermentado e após 16 - 72 horas, o café é levado para um terreiro suspenso.

Fonte: Do autor (2023).

2.8 Uso de Cultivos Iniciadores

Os microrganismos responsáveis pela fermentação do café (leveduras e bactérias do ácido lático) podem desempenhar uma série de papéis, como degradação da mucilagem (atividade pectinolíticas), inibição do crescimento de fungos produtores de micotoxinas e produção de componentes ativos de sabor (DA MOTA *et al*., 2020; DE SOUZA *et al*., 2017). Além disso o uso de culturas iniciadoras (principalmente linhagens de leveduras), surgiu nos últimos anos como uma alternativa promissora para controlar o processo de fermentação e promover o desenvolvimento da qualidade do café, fornecendo maior controle e consistência modificando os precursores de sabor e perfis de voláteis com caraterísticas desejáveis (EVANGELISTA *et al*., 2014; LEE *et al*., 2017; SILVA *et al*., 2013).

As fermentações controladas em cafés são utilizadas a fim de obter maior qualidade e evitar o crescimento de microrganismos indesejáveis como *Aspergillus spp* (produtor de ocratoxina A) (POLTRONIERI; ROSSI, 2016). A adição de starters no processo fermentativo pode apresentar comportamentos diferentes dependendo de diferentes fatores como o tipo de café, o estado de maturação, o método de processamento, condições ambientais como temperatura, metros de altitude, resultando em cafés com caraterísticas sensoriais diferentes e positiva comparados com cafés sem utilização de starters (BRESSANI *et al*., 2018; EVANGELISTA *et al*., 2014; MARTINS *et al*., 2019; RIBEIRO *et al*., 2017).

Apesar dos benefícios da fermentação do café, esse processo deve ser monitorado uma vez que, quando mal executada, a fermentação pode resultar em sabores indesejáveis (azedo) (POLTRONIERI; ROSSI, 2016). O monitoramento do processo fermentativo (temperatura, pH, sólidos solúveis) ajuda a conhecer o desenvolvimento do processo, já que o aumento da temperatura é um indicador do aumento da população microbiana, e a diminuição do pH junto com a estabilidade da temperatura são indicadores do final do processo (DA MOTA *et al*., 2020; JIMENEZ *et al*., 2023; MARTINS *et al*., 2020).

2.9 Respostas metabólicas dos grãos de café durante o processamento

Durante o processamento pós-colheita do café são desenvolvidas alterações bioquímicas importantes nos grãos de café, relacionadas ao metabolismo da germinação, cuja extensão depende do método de processamento, seja úmido ou seco (SELMAR; KLEINWÄCHTER; BYTOF, 2015; TAVEIRA *et al*., 2015).

Taiz *et al*. (2017a) descrevem como as sementes iniciam um processo de dormência que pode ser provocado por conta de inibidores internos (ácido abscísico - ABA) que impedem o desenvolvimento do processo germinativo, denominado dormência primária. O ABA é um

inibidor natural da germinação no fruto do café, que dependendo do tipo de processo pós-colheita, ele pode ser eliminado de forma mecânica, o que facilita o início do processo germinativo dependendo do método de processamento (SELMAR *et al*., 2006). Além do ABA as condições não favoráveis que inibem a germinação por um período conhecido como dormência secundária, é aquela dormência fisiológica imposta ao embrião pela casca da semente e por outros tecidos envolventes, como endosperma, pericarpo ou órgãos extraflorais, é conhecida como dormência imposta pela casca, a qual tem uma limitação mecânica da dormência (TAIZ *et al*., 2017a).

Além disso, para que os processos metabólicos das sementes possam iniciar são necessárias diferentes condições ambientais (a_w, temperatura, oxigênio) assim como condições físicas que eliminem os hormônios presentes naturalmente no café que inibem estes processos (ABA), no entanto o processamento pós colheita (seco ou úmido) rompem a casca e eliminam alguns desses inibidor facilitando o início dos processos metabólicos da germinação (FERREIRA *et al*., 2018; SELMAR *et al*., 2006; TAIZ *et al*., 2017b).

2.9.1 Atividade enzimática durante a pós-colheita do café

Existem diferentes enzimas envolvidas na proteção e manutenção do embrião e das camadas protetoras (membranas) das reservas energéticas (esterase, catalase, peroxidase entre outras) (SANTOS *et al*., 2021), assim como enzimas chaves necessárias para o início do metabolismo germinativo (entre estas: isocitrato liase, beta tubulina, endo beta mananase) (SELMAR *et al*., 2006; TAIZ *et al*., 2017c). Durante os processos fermentativos dependendo do método de processamento (natural, CD ou úmido) alterações mecânicas (retirada da casca), assim como dano nas membranas celulares, aceleram a perda de hormônios naturais inibidores da germinação (ABA e a permeabilidade da casca), que junto com condições ambientais (a_w, temperatura, oxigênio) permitem o início de processos metabólicos germinativos assim como consumo de reservas energéticas, oxidação, morte do embrião (COELHO *et al*., 2015; JIMENEZ *et al*., 2023; SELMAR; KLEINWÄCHTER; BYTOF, 2015; TAVEIRA *et al*., 2015). A presença de algumas enzimas catalase, oxidase, peroxidase, mostram a atividade de manutenção, início de processos oxidativos, assim como reação a ambientes estressantes (anaerobioses no caso do álcool desidrogenase) que podem resultar em perda de reservas energéticas pelas rotas metabólicas desenvolvidas nos processos metabólicos de respiração e manutenção da semente, assim como dano ou morte do embrião (ŠVUBOVÁ *et al*., 2021; TAIZ *et al*., 2017a).

3 CONSIDERAÇÕES GERAIS

O processamento pós-colheita é uma das etapas essenciais na produção de café e, juntamente com uma boa seleção de genética, manejo e processamento, nos ajuda a produzir cafés com boa qualidade química e sensorial. Na atualidade a utilização de diferentes métodos de processamento (natural, despolpado e úmido) junto com a utilização de novas tecnologias no processo de fermentação (SIAF, com ou sem inoculação de microrganismos) assim como o monitoramento constante dos processos, pode permitir um processamento padronizado, assegurando boa qualidade do café.

Os metabolismos germinativos da semente do café são desenvolvidos durante o processo de fermentação do café, e estes podem provocar câmbios nas reservas energéticas no interior da semente, além disso a fermentação do café por SIAF provoca uma diminuição na viabilidade e morte da semente. No entanto esta diminuição da viabilidade da semente produzida durante a fermentação não é diretamente relacionada com uma diminuição da qualidade do café. Já que durante este processamento existe difusão de compostos orgânicos ao interior da semente (açúcares, compostos voláteis, álcoois, aminoácidos entre outros) potencializando a qualidade sensorial do café.

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SEGUNDA PARTE: ARTIGOS

ARTIGO 1: Influence of anaerobic fermentation and yeast inoculation on the viability, chemical composition, and quality of coffee

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Abstract

Microbial metabolites produced during fermentation migrate into the coffee and can influence the decrease in seed viability and coffee quality. This study evaluated the effects of physiological changes in seed viability on the sensory quality of the beverage using starter cultures through self-induced anaerobic fermentation (SIAF) in *Coffea arabica* L. for natural and pulped coffee. The yeasts were monitored by real-time polymerase chain reaction (qPCR). High-performance liquid chromatography (HPLC) detected citric, malic, and succinic acids in all fermented coffees. Furthermore, lactic acid was mainly identified in those coffees processed by the SIAF method. Volatile compounds (40) were detected by gas chromatography-mass spectrometry (GC-MS). Alterations in physiological quality were identified, with decreased embryonic viability and cell membrane damage by tetrazolium and electrical conductivity tests. All fermented coffees obtained scores above 80 points. The *Torulospora delbrueckii* yeast got the best score (86.50) in pulped coffee, and the *Candida parapsilosis* yeast received the highest score (85.90) in the natural coffee using the SIAF method. The coffees were characterized by aromas and flavors of citrus, caramel, honey, chocolate, and chestnut. The coffee fermentation process with yeast inoculation affected the coffee bean viability but not the beverage's sensory quality, indicating that the use of yeast starters by SIAF favors the production of specialty coffees with differentiated sensory characteristics.

Keywords: Coffee fermentation, Specialty coffees, Seed physiological, Yeast starter, Sensory quality.

1. Introduction

Specialty coffees have changed international trade in recent years, from selling regular coffee to a special product (Sittipod et al., 2019), causing an increase in their popularity in global markets and driving the search for new postharvest technologies that help the production of specialty coffees (Córdoba et al., 2021). Traditionally, postharvest processing of coffee is carried out using natural, pulped, and wet methods, which are considered responsible for some differences observed in the sensory quality of coffee (Nadaleti et al., 2022; Selmar et al., 2014). During postharvest processing, fermentation occurs spontaneously by epiphytic microorganisms (bacteria, yeasts, and fungi), which process the coffee mucilage (Silva et al., 2013). The production of microbial metabolites can reach the interior of the seed, thus leading to beneficial (organic acids of interest, esters, alcohols, sugars) or harmful effects (undesirable organic acids and toxins) on the quality of coffee beans (Elhalis et al., 2021; Hadj Salem et al., 2020; Wang et al., 2019; 2022). In addition, during the postharvest period, physiological processes associated with germination begin inside the seed (respiration and cell division). As a result, they can use energy reserves, putting the embryo's viability at risk and causing the risk of a negative impact on the sensory quality of the coffee beverage (da Silva et al., 2019; Kitzberger et al., 2020; Selmar et al., 2006, 2014).

When the coffee fruit reaches maturity, the seed is ready to begin germinating. However, this metabolic process is inhibited by a dormancy process caused by natural inhibitors (abscisic acid), the shell (a protective barrier that creates an impenetrable environment), and the lack of environmental conditions (moisture, oxygen, light, and temperature) (Taiz et al., 2017). During the postharvest period, the start of the metabolic processes of germination may be linked to the appropriate conditions and the elimination of natural inhibitors present in coffee (Selmar et al., 2006; Taiz et al., 2017).

The use of starter cultures (mainly yeast strains) has emerged as a promising option to improve the quality and modify the sensory profile of coffee (Cassimiro et al., 2022; Elhalis et

al., 2021; Mahingsapun et al., 2022). These microbial starters may be used in closed bioreactors, thus creating an anaerobic environment that allows for self-induced anaerobic fermentation (SIAF) (da Mota et al., 2022; Pereira et al., 2022). The SIAF method consists of gradual CO_2 production driven by microbial metabolism, improving the fermentative performance of lactic acid bacteria (LAB) and yeasts and increasing the production of metabolites (organic acids, alcohols, and volatile composts) that intensify the aromas and flavors desired in coffee (Cassimiro et al., 2022; Martinez et al., 2019).

Consequently, the present study aimed to evaluate the effects of decreased seed viability on the sensory quality of the coffee beverage for natural and pulped postharvest coffee (*Coffea arabica* L. variety Topázio Amarelo) processed using starters (*Saccharomyces cerevisiae* CCMA0543, *Candida parapsilosis* CCMA0544, and *Torulospora delbrueckii* CCMA0684) by SIAF compared with that of coffee processed by a SIAF control and a conventional process (without bioreactors) through physiological, real-time polymerase chain reaction (qPCR), chemical and sensory analysis.

2. Materials and Methods

2.1. Coffee

Coffee cherries (*Coffea arabica* L.) of the 'Topazio Amarelo' variety were harvested at an altitude of 850 meters above sea level at the farm Cafés Monte Alegre (S 1°59'56", W 54°4'58") in Alfenas, Minas Gerais (M.G.), Brazil.

2.2. Yeast

The *Saccharomyces cerevisiae* (CCMA0543), *Candida parapsilosis* (CCMA0544), and *Torulaspora delbrueckii* (CCMA0684) yeasts belonging to the Agricultural Microbiology Culture Collection (CCMA) of the Department of Biology at UFLA, Lavras, M.G., Brazil, were

selected as starter cultures for their ability to survive throughout the fermentation process and for generating chemical and sensory changes that improve coffee quality (Silva et al., 2013). The yeasts stored at -80°C were reactivated and grown according to Martins et al. (2022). Cells were recovered by centrifugation (3200 × g; 10 min) and resuspended in sterile water (500 mL) until reaching a concentration of 10^7 and 10^8 cells/mL in coffee.

2.3. Coffee fermentation processing

The natural and pulped coffee (40 L) was processed without and with yeast inoculation in high-density polyethylene cylindrical bioreactors with a 50 L capacity using the method of SIAF (da Mota et al., 2022; Pereira et al., 2022). Five treatments were performed: conventional processing (the traditional process where the coffee is harvested and then dried in the sun on cement platforms, with the addition of pulping for pulped coffee), SIAF control (without inoculation), *S. cerevisiae* (CCMA0543), *T. delbrueckii* (CCMA0684), and *C. parapsilosis* (CCMA0544). All the treatments were carried out in triplicate. During fermentation, the temperature and humidity of the environment and the temperature and concentration of coffee solids (% TSS) were monitored until stabilized (Table 1) (da Mota et al., 2022; Pereira et al., 2022). The fermentation time for both natural and pulped coffee was 180 h, and the end of fermentation occurred by decreasing the coffee temperature (Table 1). Samples were taken at the beginning of fermentation (0 h) and at the end of drying (480 h). The coffee was dried immediately in the sun on suspended terraces until reaching 11% moisture.

2.4. Monitoring of inoculated yeast populations

The inoculated yeast population was monitored by qPCR using a QIAamp DNA Mini Kit (Qiagen, Hilden Germany) to extract DNA from sample strains at the end of processing (480 h). All yeast species were cultured separately on YEPG agar at 28°C for 24 h. They were serially

diluted (1:10) from 10^8 to 10^3 cells/mL, measuring each point of the standard curve in triplicate for use in qPCR (Batista et al., 2015). For this analysis, it was necessary to use specific primers (Supplementary Table S.1) for each yeast species.

2.5. Analysis of chemical compounds

2.5.1. Determination of organic acids by high-performance liquid chromatography (HPLC)

Organic acids (acetic, citric, lactic, malic, oxalic, succinic, and tartaric) were evaluated by HPLC (Shimadzu Corp., Japan) with a UV detector at 210 nm. The natural and pulped coffee was evaluated at the beginning and end of fermentation (0 and 480 h). The operating conditions were described by Evangelista et al. (2014). For each sample, ten grams of coffee was mixed twice with 10 mL of Milli-Q water for 5 minutes, and the solutions (20 mL) were centrifuged at 12,745 × g for 10 minutes at 4°C. The pH value of the samples was adjusted to 2.11 with perchloric acid and centrifuged again. Then, the supernatant was filtered through 0.22 μm cellulose acetate membranes. The samples obtained were stored at -18°C until analysis.

2.5.2. Analysis of volatile compounds by headspace/solid-phase (HS-SPME) microextraction/gas chromatography/mass spectrometry (GC/MS)

Volatile compounds were extracted from roasted coffee and green coffee at the end of postharvest processing using a manual headspace solid-phase microextraction (HS-SPME) procedure and a 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane SPME fiber (Supelco Co., Bellefonte, PA., USA) (Evangelista et al., 2014). Two grams of coffee from each treatment were ground with liquid nitrogen and placed in a 15 mL hermetically sealed flask. After equilibration at 60°C for 15 min, the volatile compounds were extracted at 60°C for 30 min. Injections were performed by fiber exposition for 2 min. A GCMS-QP2010 (Shimadzu) equipped with mass spectrometry (MS) and a silica capillary Carbo-Wax 20 M (30 m×0.25 mm

x 0.25 mm) column were used for GC/MS analysis. The oven temperature was held at 60°C for 5 min, followed by a gradient from 60°C to 230°C at 10°C/min, and held at 230°C for 15 min. The carrier gas (He) was used at a flow rate of 1.95 mL/min. The volatile compounds were identified by comparing the mass spectra to the NIST11 library. In addition, an alkane series (C10–C40) was used to calculate each compound's retention index (RI) and compare them to the scholarly literature's RI values.

2.6. Tetrazolium test

Fifty seeds from each fermentation protocol with four replicates were soaked in water (36 h). The embryos were then extracted and kept in a polyvinylpyrrolidone (PVP) antioxidant solution until submerged in a 0.5% 2, 3, 5 triphenyl chloride tetrazolium solution in dark flasks at 30°C for 2 h. Finally, a longitudinal cut was made in the middle of the embryos to perform the viability analysis of the embryo with a 10x stereoscopic magnifying glass to visualize its internal and external structures, better classifying them as viable and nonviable according to the location and extension of the stained areas (Clemente et al., 2012).

2.7. Electrical conductivity (E.C.)

Four replicates of 25 seeds of each fermentation treatment were first weighed and then soaked in containers with 37.5 mL of deionized water, keeping them in BOD at a constant temperature of 25°C for 24 h. After that period, the electrical conductivity of the solution containing the seeds was read in $\mu S.cm^{-1}.\ g^{-1}$ (Malta et al., 2005).

2.8. Sensory evaluation

Five trained coffee tasters with Q-Grader certificates evaluated the coffee samples, following the sample preparation protocol of the Specialty Coffee Association. The following

attributes were evaluated: fragrance, aroma, flavor, aftertaste, acidity, body, uniformity, balance, sweetness, cleanliness, defects, and general score according to the SCA Cupping Protocol (SCA, 2018, p. 14).

2.9. Statistics

The treatments were applied through a 5x2 mixed factorial design (yeast x processing method) to evaluate sensory analysis, organic acids, electrical conductivity, and tetrazolium test. The tests for organic acids, electrical conductivity, and tetrazolium were also compared before (0 h) and after the fermentation process (480 h) using Student's t test. A 3x2 mixed factorial design (yeast x processing method) was used for qPCR analysis at the end of processing (480 h) to monitor the inoculated yeast population. An analysis of variance (ANOVA) was performed using the Scott Knott test with a 5% significance level ($p \leq 0.05$) and with a significance level of $p \leq 0.088$ for the tetrazolium test, using the Sisvar software version 5.6 (Ferreira, 2014). In addition, a principal component analysis (PCA) was performed for the volatile compounds using the SensoMaker program v. 1.92 (UFLA, Lavras, Brazil) (Pinheiro et al., 2013).

3. Results

3.1. Population of yeasts by qPCR

The population of *S. cerevisiae, C. parapsilosis,* and *T. delbrueckii* yeasts at the end of processing was evaluated by qPCR (Figure 1). The inoculated yeasts grew and maintained viability during fermentation. The growth of the *C. parapsilosis* population was not stimulated by using the SIAF method, and it was minimally present in the conventional process. In inoculated fermentation using SIAF, a higher population of *T. delbrueckii* (8.10 $\log_{10}$ cells/g) was observed in the natural coffee, while *S. cerevisiae* (8.21 $\log_{10}$ cells/g) showed the highest

population in pulped coffee. The inoculated fermentation with *T. delbrueckii* reached 8.10 log_{10} cells/g, followed by *S. cerevisiae* with 7.71 log_{10} cells/g for the natural coffee method. In the pulped method, the treatment inoculated with *S. cerevisiae* had the highest population with 8.21 log_{10} cells/g, followed by *T. delbrueckii* with 7.31 log_{10} cells/g.

In addition, the monitoring of the yeasts in the SIAF control treatment showed that the yeast *S. cerevisiae* obtained the highest presence in the coffees without inoculation, with 6.98 log_{10} cells/g in the natural coffee and 7.89 l log_{10} cells/g in the pulped coffee. Followed by the *T. delbrueckii* yeast with 7.21 log_{10} cells/g in natural coffee and 6.93 log_{10} cells/g in pulped coffee, and the yeast with the lowest population present was *C. parapsilosis* with a population of 3.32 log_{10} cells/g in natural coffee and 2.98 log_{10} cells/g in pulped coffee.

The conventional processing was the treatment with the lowest yeast population in both methods. For example, the yeast *S. cerevisiae* with 2.44 log_{10} cells/g obtained the highest population in the natural coffee, and the yeast *C. parapsilosis* with 2.92 log_{10} cells/g had the highest population in the pulped coffee.

3.2. Evaluation of organic acids

The acids (acetic, citric, lactic, malic, oxalic, succinic, and tartaric) are present in the coffee at the beginning (freshly harvested coffee before fermentation for 0 h) and at the end (end of drying for 480 h) of processing were evaluated in natural and pulped coffees. At the beginning of the processing (freshly harvested coffee), citric, malic, and succinic acids were identified (Table 2); oxalic, tartaric, lactic, and acetic acids were not identified.

The acids concentration showed significant differences ($p \leq 0.05$) between the natural and pulped coffee processing, however, no difference was obtained among fermentations. For example, citric acid was detected in high concentrations in natural (4.85 g/kg) and pulped (3.01 g/kg) coffee at the beginning of the process (0 h).

By the end of processing, citric, malic, succinic, and lactic acids were detected, and high lactic acid production was observed in coffees fermented by SIAF (Table 2). In addition, natural coffee had a higher production of acids (citric, malic, succinic, and lactic acids) than pulped coffee in the treatments processed by SIAF. On the other hand, conventional processing showed higher production of acids (citric, malic, succinic, and lactic acids) in pulped coffee than in natural coffee. Significant differences ($p \leq 0.05$) were found in acid production between the treatments. *T. delbrueckii* had the highest citric acid production, with 7.29 g/kg in natural coffee and the conventional process with 4.04 g/kg for pulped coffee. For succinic acid, the SIAF control presented the highest concentration with 2.38 g/kg in natural coffee and in pulped coffee conventional processing with 1.60 g/kg. For lactic acid, *S. cerevisiae* was 12.15 g/kg in natural coffee, and *C. parapsilosis* was 4.36 g/kg in pulped coffee. Moreover, conventional processing obtained the highest yields for malic acid, with 2.02 g/kg in natural coffee and 2.75 g/kg in pulped coffee.

3.3. Volatile compounds

A total of 40 volatile compounds were detected between green (14) and roasted (39) coffees (Supplementary Table S.2). These compounds were classified into 14 groups: pyrroles (6), furans (6), esters (5), acids (4), alcohols (3), ketones (3), pyrazines (3), pyridines (2), lactones (2), aldehydes (2), phenols (1), pyrans (1), thiophenes (1) and others (1) (Flamet, 2001). Ethyl oleate, octadecanoic acid, and ethyl ester were detected only in coffee processed by natural coffee in green and roasted coffee. Principal component analysis (PCA) analyzed the GCMS results and correlated the volatile compounds detected with each treatment and processing method. Figure 2 shows the PCA results, with numbers (1) for green coffee and (2) for roasted coffee and letters (A) for natural and (B) for pulped coffee. In green coffee, PC1 and PC2 explained 79.41% and 77.49% of the total variance for natural and pulped coffee, respectively.

In natural green coffee, there is a relationship between *C. parapsilosis* yeasts and the compounds linoleic acid ethyl ester, hexanoic acid, 2-methylbutyl ester, hexadecanal, and n-nonadecanol, and between SIAF control 2-pentadecanone, the conventional process with compound pentadecanal and *S. cerevisiae* and *T. delbrueckii* do not show a direct correlation with any specific compound.

In roasted coffee, PC1 and PC2 explained 77.77% and 77.79% of the total variance for natural and pulped coffee, respectively. In natural coffee, the SIAF control was related to Compounds 2-thiophenemethanol, ethanone, 1-(1H-pyrrol-2-yl), and 1H-pyrrole, 1-(2-furanylmethyl). The conventional process with compounds 3- pyridinol and 5-hydroxymethylfurfural, *C. parapsilosis* with compound phenylethyl alcohol, *T. delbrueckii* with 1H-pyrrole-2-carboxaldehyde, 2- pentadecanone, and decanoic acid, and *S. cerevisiae* did not show a direct correlation with any specific compound.

In pulped green coffee, SIAF control is related to hexanoic acid, 2-methylbutyl ester, and the conventional process is related to caffeine, *T. delbrueckii*, *C. parapsilosis* and *S. cerevisiae* did not present a correlation. A correlation was observed between *C. parapsilosis* yeast and the SIAF control for pulped roasted coffee. The SIAF control showed a correlation with the Compounds 2(5H)-furanone, 4-methyl-5H-furan-2-one, 2-thiophenemethanol, 1H-pyrrole-2-carboxaldehyde, and benzyl alcohol, *C. parapsilosis* with 3-methyl-2-pyrazinylmethanol, 2-furanmethanol, ethanone, 1-(1H-pyrrol-2-yl), 2-heptadecanone, 1H-pyrrole, 1-(2-furanylmethyl), 1H-pyrrole-2-carboxaldehyde, 1-methyl, indole, and ethanone, 1-(2-furanyl), *S. cerevisiae* with n-hexadecanoic acid, and the conventional process, and *T. delbrueckii* did not show any correlation.

3.4. Tetrazolium test

Table 3 shows the results of the tetrazolium test, with significant differences ($p \leq 0.088$)

between treatments and processing methods and between the times of freshly harvested coffee before fermentation (0 h) and coffee at the end of drying (480 h).

In general, we can say that the pulped coffee processing method has caused less embryonic damage than natural coffee processing. However, only the treatment of natural coffee conventionally obtained the same results as coffee before treatments (0 h).

3.5. Electrical conductivity (E.C.)

Before processing, at time 0, a statistically significant difference ($p \leq 0.05$) was found between the processing methods (Table 4). At the end of processing (480 h), the results showed statistically significant differences ($p \leq 0.05$) between processing methods and between treatments. The conventional processing obtained the lowest electrical conductivity values in the natural (259.53 $\mu S.cm^{-1}. g^{-1}$) and pulped (209.00 $\mu S.cm^{-1}. g^{-1}$) methods, indicating the better physiological quality of coffee by this treatment.

3.6. Sensorial analysis

Sensory analysis showed that all coffees scored above 80 and were classified as specialty coffees. When inoculated with *T. delbrueckii* (86.50), pulped coffee obtained the best scores; natural coffee showed better results with *C. parapsilosis* (85.90). The coffees processed by the conventional method obtained the lowest scores (83.75 for natural coffee and 84.25 for pulped coffee). Figure 3 shows the intensity of sensory attributes (sweetness, acidity, body, astringency, bitterness, and finish) that contributed to the final score for each treatment. Likewise, these coffees were characterized by aromas and flavors of citrus fruits, caramel, honey, chocolate, and chestnuts for natural coffee and flavors and aromas of citrus fruits, nuts, chocolate, caramel, and chestnuts for pulped coffee. In addition, the Q-graders detected aromas and fermented flavors (of wine) in the natural coffees processed by SIAF.

4. Discussion

SIAF is a new technology applied to the fermentative processing of coffee in closed bioreactors, creating a modification in the fermentation environment by limiting the availability of oxygen and allowing the development of facultative aerobic microorganisms (Cassimiro et al., 2022; da Mota et al., 2022). The anaerobiosis caused by SIAF generates a decrease in cellular respiration in the seed, forced to carry out anaerobic respiration, which translates into greater consumption of nutrients to produce the energy necessary for maintenance and development functions, affecting the viability of the seed (Taiz et al., 2017). Kleinwächter and Selmar, (2010) demonstrated that the anaerobic process intensifies the consumption of sugars by the seed. Therefore, microbiological interactions are created that favor the development of yeasts through different metabolic pathways (Feldmann, 2012; Takagi et al., 2015). However, the growth of microorganisms during fermentation generates physicochemical changes in the beans, loss of water, and changes in organic compounds (acids, sugars, volatile) that provide additional flavors and aromas through the metabolites produced in this process, which can migrate to the bean, producing different flavors and aromas that favor the quality of a beverage (Evangelista et al., 2014; Silva et al., 2013).

The use of yeast starter cultures (*C. parapsilosis, T. delbrueckii,* and *S. cerevisiae*) during coffee fermentation process has been used in different investigations (da Mota et al., 2022; Martins et al., 2022) showing the yeast's ability to carry out fermentation and remain viable until the end of the drying process. In addition to guaranteeing the sensory quality of the beverage through the production of the desired sensory attributes.

Epiphytic microorganisms participate in coffee fermentation, competing for available nutrients (pectin, sugars, amino acids, polysaccharides) among themselves and with starter cultures (Silva et al., 2013). The microbial metabolites produced can migrate to the interior of

the bean, altering the internal chemical composition of the bean and providing organic compounds (amino acids, sugars, volatile compounds) that modify the sensory profile of the coffee (Hadj Salem et al., 2020; Pereira et al., 2022; Wang et al., 2022).

At the end of coffee processing, the inoculated yeasts (*S. cerevisiae, C. parapsilosis*, and *T. delbrueckii*) maintained populations at least 1 log above the populations found in the SIAF control and conventional processing. These yeasts were identified both in the SIAF control and in the conventional processing, which was expected, as these yeasts are naturally in coffee in populations between 10^2 and 10^6 cells/mL (Silva et al., 2013; Vilela et al., 2010). Through the generation of suitable environments for the growth of these microorganisms (presence and absence of oxygen), environmental conditions were different from the conditions outside the bioreactors (Cassimiro et al., 2022; da Mota et al., 2022), observing differences between the treatments in bioreactors and the conventional processing that was directly the terraces. *T. delbrueckii* and *S. cerevisiae* exceeded six $\log_{10}$ cells/mL in the SIAF control and four $\log_{10}$ cells/mL above conventional processing, thus demonstrating that SIAF favors the growth of these microorganisms.

Coffee fruits naturally contain organic acids in their composition, such as citric, malic, and succinic acids, which influence the perceived acidity of the beverage (Bressani et al., 2021). However, acid degradation occurs during coffee fermentation, as well as an increase in their concentration, due to the metabolism of microorganisms (Ribeiro et al., 2017). In complex fermentative processes, these acids are used as a carbon source by the microbiota present, such as in the fermentation of citric acid by lactic acid bacteria (LAB), increasing or decreasing its concentration during coffee processing as a product generated by the metabolism of microorganisms (Martinez et al., 2019). These compounds will influence the perceived acidity in coffee, an essential attribute of coffee quality combined with sweetness, bitterness, and aroma, which improves the beverage's sweetness (Sunarharum et al., 2014). Therefore,

producing organic acids during coffee fermentation plays a promising role in creating specialty coffees with desired sensory characteristics (Cassimiro et al., 2022; Wang et al., 2019).

Lactic acid, absent at the beginning of processing, becomes the main acid during fermentation, with the highest quantification compared to the other acids detected. Similar results were reported by da Mota et al. (2022). This lactic acid in the coffee is related to the decarboxylation of the malic acid by LAB, with a decrease in malic acid observed compared to that detected at the beginning of processing (Cassimiro et al., 2022; Leeuwenhoek, 1999). Furthermore, citric, malic, succinic and lactic acid in green beans affect the formation of volatile compounds during roasting, such as pyrazines, furans, and esters (Elhalis et al., 2021).

The volatile compounds identified in green coffees, alcohols, esters, ketones, aldehydes, and caffeine, are responsible for aromas characteristic of coffee with aromas of citric fruits, nuts, and florals (Flamet, 2001; Sunarharum et al., 2014), indicating that the coffees were free of defects (rotted, over-fermented, brocaded, moldy coffees) (Elhalis et al., 2021). Roasted coffees had an increased number of volatile compounds due to the several reactions produced during roasting (Maillard reaction, Strecker degradation) (Flamet, 2001; Prakash et al., 2022). Thirty-nine compounds detected in 14 classes were dominated by seven classes: Pyrroles (7, provide sweet, smoky, herbaceous, mushroom, woody aroma), Furans (6, provide the characteristic aroma and flavor of coffee, sweet, fruity, nutty, caramel), Esters (4, fruity flavors, original notes and flavors produced by fermentative metabolites), Acids (4, contribute to the characteristic aroma of the coffee, contribute to the quality of coffee, characteristic fruity flavors), Alcohols (3, characteristic flavors of coffee, enhance the aroma and flavor of coffee), Ketones (3, characteristic aromas of coffee), Pyrazines (3, aromas and flavors characteristic of roasted coffee, chocolate, roasted walnuts, roasted peanuts, hazelnuts, popcorn, herbal) (Flamet, 2001; Prakash et al., 2022).

When evaluating coffee's seed vigor and cell membrane integrity before and at the end of

processing through tetrazolium and electrical conductivity tests, a decrease in the viability of seed was observed, except for conventional processing in natural coffee, which maintained the same viability as freshly harvested coffee. Bytof et al. (2007) and Selmar et al. (2006) describe the function of the husk as a protective barrier that avoids the beginning of metabolic processes within the seed, preventing the consumption of energetic reserves. All the coffees processed by SIAF decreased their physiological quality, with statistically significant differences ($p \leq 0.088$) between the treatments and the processing. The decrease in the viability of the embryos observed by the tetrazolium test is influenced by the metabolic reactions of microorganisms and the beginning of the metabolic processes of the seed during fermentation (Selmar et al., 2014). These metabolic processes (cellular respiration, cell division, and cell maintenance processes, among others) can use the nutritional reserves inside the bean, transforming them into other compounds. It is also possible that the decrease in available oxygen caused by SIAF forces the seed to carry out cell breathing by anaerobic processes, which results in greater nutrient consumption (reserves) for energy production (Pereira et al., 2022; Selmar et al., 2014; Taiz et al., 2017). For the conventional processing of pulped coffee, the behavior was similar to that of the treatments processed by SIAF, possibly due to the loss of protection from the husk to the coffee bean (Selmar et al., 2008, 2014).

Similar electrical conductivity results were observed with statistically significant differences ($p \leq 0.05$) between treatments and processing methods. This test can show the rupture of the cell membrane and the loss of control of its permeability, by indirect evaluation of the degree of structuring of the cell membrane, by determining the number of ions leached in the soaking solution, such as sugars, amino acids, organic acids, proteins and phenolic substances, and inorganic ions, such as K^{+}, Ca^{+2}, Mg^{+2}, $Na^{+,}$ and Mn^{+2} (Marcos Filho, 2015). These tetrazolium and electrical conductivity tests showed that the physiological quality (theoretical capacity of the seeds to express their vital functions in favorable and unfavorable

environmental conditions) of the fermented coffees began to decrease (Marcos-Filho, 2015; Pazmiño-Arteaga et al., 2022).

The aroma and flavor were different for each treatment, showing different behavior for each yeast, as observed in other studies (Bressani et al., 2021; Evangelista et al., 2014; Martinez et al., 2019; Martins et al., 2019). Notes of citrus fruits, caramel, honey, chocolate, and chestnuts were perceived in all the treatments that adopted the natural method and notes of fermentation (wine) for those processed by SIAF. In addition, the SIAF control produced a coffee beverage with a wood flavor, which is considered a defect (Flamet, 2001). The pulped coffee was characterized by flavors and aromas of citrus and nuts, chocolate, caramel, and chestnuts. These sensory characteristics are influenced by volatile composition, such as, benzyl alcohol related to fruit flavors, Phenylethyl alcohol with floral aromas (roses), 1h-pyrrole, 1- (2-Furanylmethyl) coffee and fruited, Pyrazine, 3-Ethyl-2,5 -Dimethyl, relating cocoa, nuts, and roasted potatoes, Butanoic acid, 4-hydroxy with sweet caramel and creamy taste (Bressani et al., 2018; Martinez et al., 2019). In addition, other compounds related to the caramelization of sugars generate flavors and aromas characteristic of coffee (bitterness, acidity, caramel, smoked, among others) (Flamet, 2001; Prakash et al., 2022).

The coffee processed by SIAF obtained the best scores and the lowest scores were observed for coffees processed by the conventional method. These results show that viability is affected by processing and starter cultures that helps to control the fermentation process and potentiate the quality of coffee. Conventional coffee processed by the natural method was the only treatment that did not show a decrease in seed viability. However, this treatment did not obtain the best sensory scores, demonstrating that the seed's viability does not influence coffee's sensory quality.

5. Conclusions

The fermentation process negatively affects the viability of the coffee seed without negatively affecting the sensory quality of the beverage. The SIAF favors the production of specialty coffees since all fermented coffees were considered specialty coffees with scores above 80. The use of yeasts as starter cultures in the processing of coffee stimulates the production of differentiated specialty coffees, producing a variation in the composition of volatile compounds and generating differentiation in the sensory characteristics of the beverage. The use of closed bioreactors by the SIAF method favors the production of lactic acid in coffee. The *T. delbrueckii* yeast obtained the best score of 86.50 for pulped coffee, and the *C. parapsilosis* yeast obtained the highest score of 85.90 for natural coffee. Natural coffee produced higher amounts of organic acids and a better distribution of volatile compounds than pulped coffee.

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Authors statement

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Declaration of competing interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi. org/10.1016/j.fbio.2022.102218.

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Figure captions

Figure 1.

S. cerevisiae (CCMA0543), *T. delbrueckii* (CCMA0684), and *C. parapsilosis* (CCMA0544) population by qPCR at the end of coffee drying (480 h). Means for microbial density at the end of coffee processing followed by the same lowercase (treatments) and uppercase (processing method) letter did not differ from each other by the Scott–Knott test ($p \leq 0.05$). Inoc = Inoculated, Cont = Control, Conv = Conventional process.

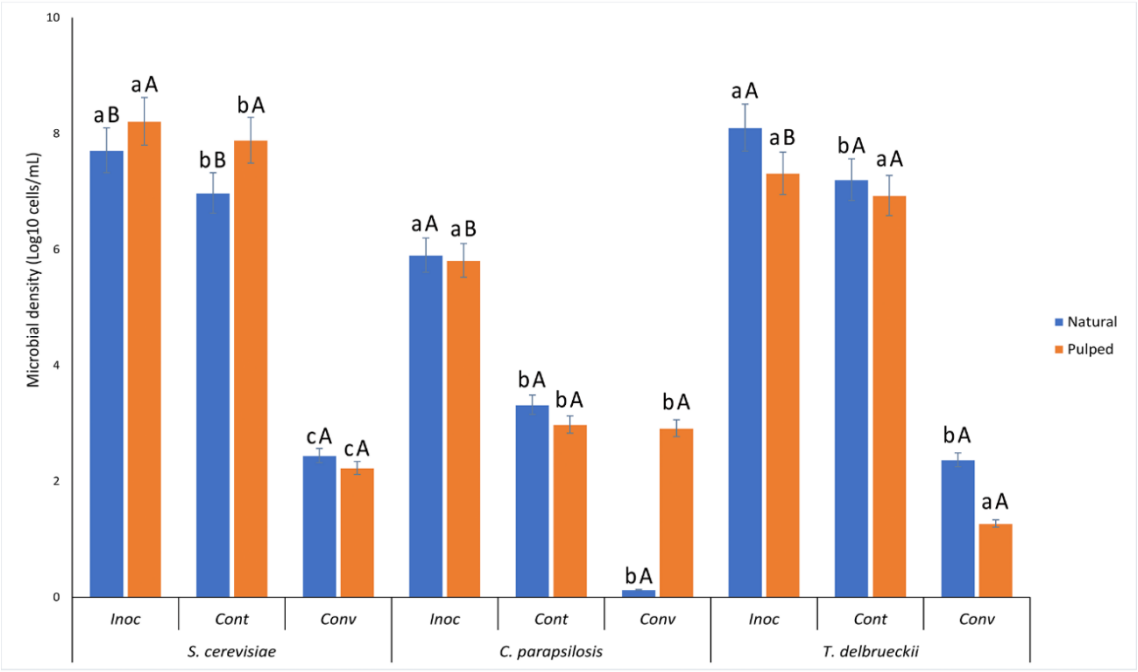

Microbial density (Log10 cells/mL)
10
8
6
4
2
0
aB
aA
bB
bA
cA
cA
aA
aB
bA
bA
bA
aA
aA
aB
bA
aA
bA
aA
Natural
Pulped
Inoc
Cont
Conv
Inoc
Cont
Conv
Inoc
Cont
Conv
S. cerevisiae
C. parapsilosis
T. delbrueckii

Figure 2. Principal component analysis (PCA) of volatile compounds detected in natural (A) and pulped (B) processes in green (1) and roasted coffees (2). Volatile compounds: (1) 1H-Pyrrole, 1-(2-furanylmethyl), (2) 1H-Pyrrole-2-carboxaldehyde, (3) 1H-Pyrrole-2-carboxaldehyde, 1-methyl, (4) 2(5H)-Furanone, (5) 2-Furancarboxaldehyde, 5-methyl, (6) 2-Furanmethanol, (7) 2-Furanmethanol, acetate, (8) 2-Heptadecanone, (9) 2-Pentadecanone, (10) 2-Pentadecanone, 6,10,14-trimethyl, (11) 2-Pyrrolidinone, 1-butyl, (12) 2-Thiophenemethanol, (13) 3-Methyl-2-pyrazinylmethanol, (14) 3-Pyridinol, (15) 3-Pyridinol, 2-methyl, (16) 4-Hydroxy-2-methylacetophenone, (17) 4-Methyl-5H-furan-2-one, (18) 5-Hydroxymethylfurfural, (19) Benzyl alcohol, (20) Butanoic acid, 4-hydroxy, (21) Caffeine, (22) Decanoic acid, (23) Ethanone, 1-(1H-pyrrol-2-yl), (24) Ethanone, 1-(2-furanyl), (25) Ethyl Oleate, (26) Furfural, (27) Hexadecanal, (28) Hexadecanoic acid, methyl ester, (29) Hexanoic acid, 2-methylbutyl ester (30) Indole, (31) 9,12-Octadecadienoic acid, ethyl ester, (32) Morpholine, 4-octadecyl, (33) n-Nonadecanol, (34) Octadecanoic acid, ethyl ester, (35) Octanoic acid, (36) n-hexadecanoic acid, (37) Pentadecanal, (38) Phenylethyl Alcohol, (39) Pyrazine, 2,6-dimethyl, (40) Pyrazine, 3-ethyl-2,5-dimethyl.

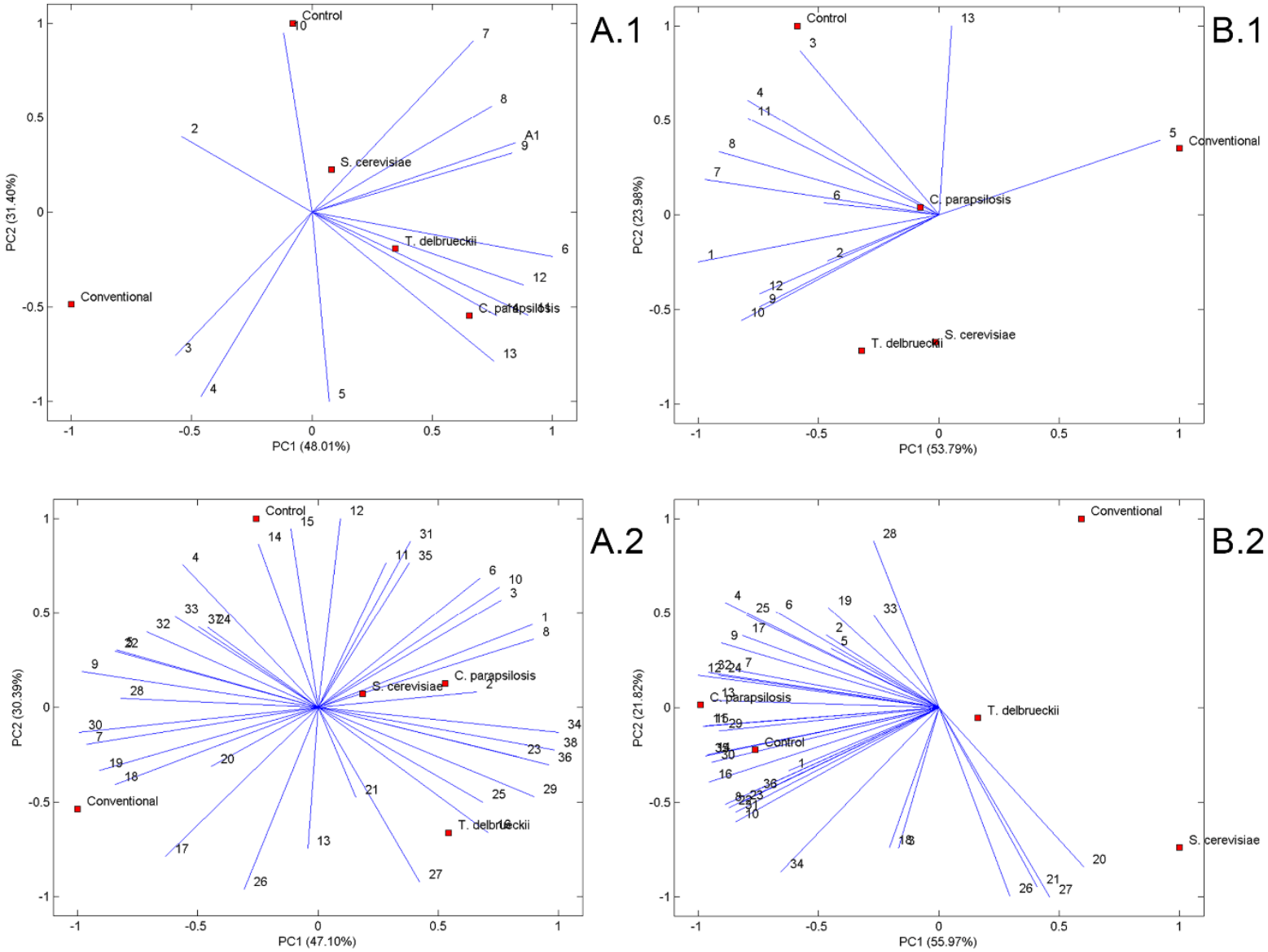
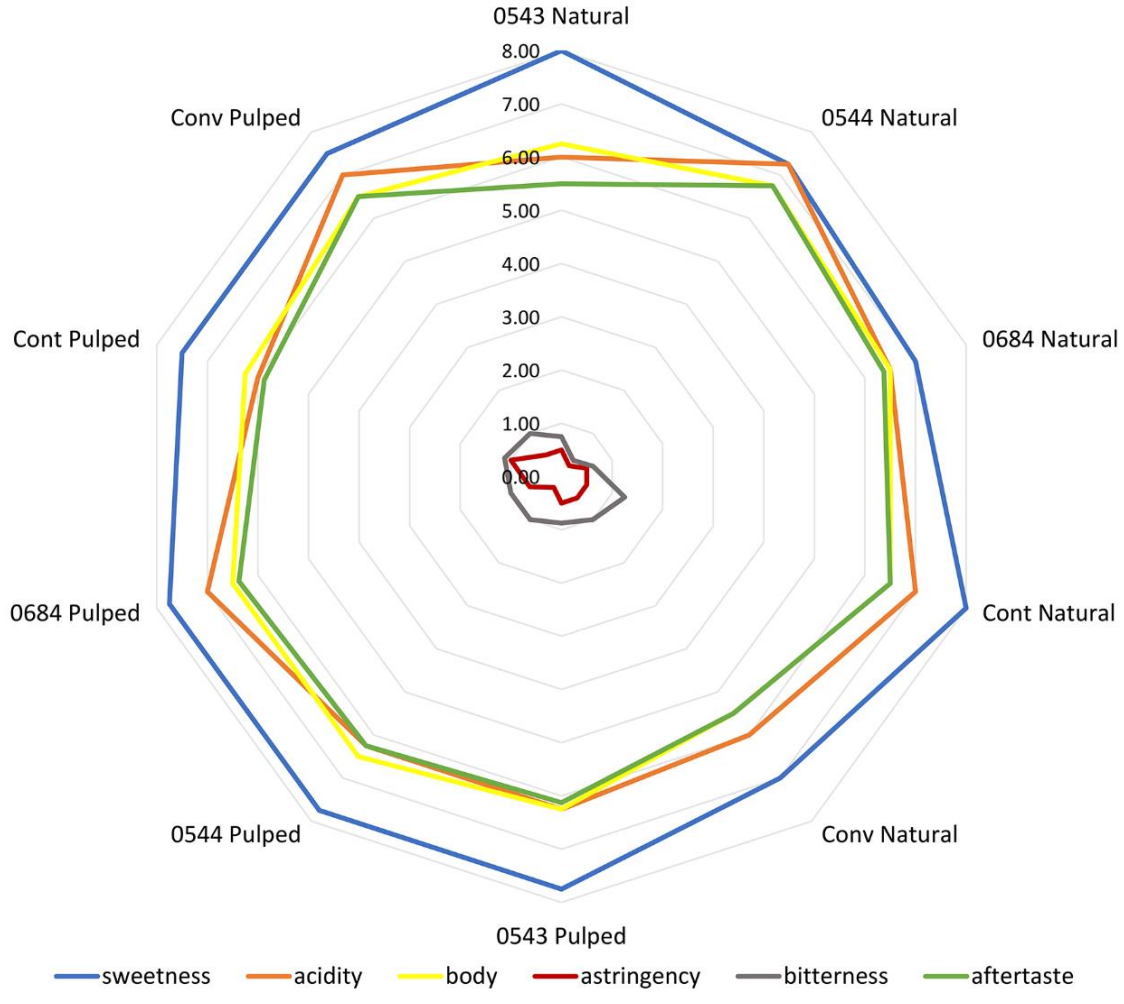

A.1
Control
S. cerevisiae
T. delbrueckii
C. parapsilosis
Conventional
A1
PC1 (48.01%)
PC2 (31.40%)
B.1
Control
Conventional
C. parapsilosis
T. delbrueckii
S. cerevisiae
PC1 (53.79%)
PC2 (23.98%)
A.2
Control
S. cerevisiae
C. parapsilosis
T. delbrueckii
Conventional
PC1 (47.10%)
PC2 (30.39%)
B.2
Conventional
T. delbrueckii
Control
C. parapsilosis
S. cerevisiae
PC1 (55.97%)
PC2 (21.82%)

Figure 3. Sensory attribute intensity. Treatments: 0543 Nat = *S. cerevisiae* Natural, 0544 Nat = *C. parapsilosis* Natural, 0684 Nat = *T. delbrueckii* Natural, Cont Nat = Control Natural, Conv Nat = Conventional processing Natural, 0543 Pulp = *S. cerevisiae* Pulped, 0544 Pulp = *C. parapsilosis* Pulped, 0684 Pulp = *T. delbrueckii* Pulped, Cont Pulp = Control Pulped, Conv Pulp = Conventional processing Pulped.



Tables

Table 1 Monitoring room temperature, moisture, coffee temperature and total soluble solids (% TSS) at 0, 96 and 180 h of fermentation.

Coffee processing	Treatments	Time (h)	Room temperature (°C)	Moisture (%)	Coffee temperature (°C)	% TSS
Natural coffee	Control (SIAF)	0	20	45	16	25
	S. cerevisiae (SIAF)	0	20	45	16	25
	T. delbrueckii (SIAF)	0	20	45	16	25
	C. parapsilosis (SIAF)	0	20	45	16	25
	Conventional process	0	19	47	15	25
	Control (SIAF)	96	19	44	19	20
	S. cerevisiae (SIAF)	96	18	45	19	18
	T. delbrueckii (SIAF)	96	18	45	19	20
	C. parapsilosis (SIAF)	96	18	46	19	18
	Conventional process	96	17	51	15	23
	Control (SIAF)	180	20	45	18	nd
	S. cerevisiae (SIAF)	180	20	45	18	nd
	T. delbrueckii (SIAF)	180	20	45	18	nd
	C. parapsilosis (SIAF)	180	20	45	18	nd
	Conventional process	180	20	45	16	nd
Pulped coffee	Control (SIAF)	0	19	47	16	26
	S. cerevisiae (SIAF)	0	19	48	16	26
	T. delbrueckii (SIAF)	0	17	48	16	26
	C. parapsilosis (SIAF)	0	17	48.5	16	26
	Conventional process	0	17	49	15	26
	Control (SIAF)	96	21	40	19	19
	S. cerevisiae (SIAF)	96	20	40	18	15
	T. delbrueckii (SIAF)	96	20	43.5	18	17
	C. parapsilosis (SIAF)	96	17	49	19	18
	Conventional process	96	17	49	16	nd
	Control (SIAF)	180	20	45	18	nd

S. cerevisiae (SIAF)	180	20	45	18	nd
T. delbrueckii (SIAF)	180	20	45	18	nd
C. parapsilosis (SIAF)	180	20	45	18	nd
Conventional process	180	18	47	17	nd

SIAF: self-induced anaerobic fermentation

Table 2. Organic acids detected (g/kg) in coffee before processing (freshly harvested 0 h) and at the end (480 h) of postharvest processing for the natural and pulping coffee.

Time (h)	Treatments	Organic acids (g/kg)

		Citric Acid		Malic Acid		Succinic Acid		Lactic Acid	
		Natural	Pulped	Natural	Pulped	Natural	Pulped	Natural	Pulped
0	Freshly harvested	4.85*	3.01*	1.13*	1.74*	0.76* B	1.17*	0.00*	0.00*
480	*S. cerevisiae* (SIAF)	3.23 dA*	2.52 bB*	0.14 bA	0.00 bA	1.49 bA	0.48 dB*	12.15 bA	4.89 aB
	C. parapsilosis (SIAF)	4.15 cA*	2.21 bB*	0.07 cA	0.00 bA	1.65 bA	0.59 dB*	8.80 dA	4.36 aB
	T. delbrueckii (SIAF)	7.29 aA	3.00 bB*	0.29 bA	0.00 bA	1.74 bA	1.21 cB	10.50 cA	1.20 cB
	Control (SIAF)	5.46 bA*	3.95 aB*	0.33 bA	0.09 bB	2.38 aA	1.60 bB	13.20 aA	3.50 bB
	Conventional process	3.13 dB*	4.40 aA*	2.02 aB	2.75 aA	1.26 bB*	2.22 aA*	0.00 eA*	0.29 dA

Means followed by the same lowercase letter (columns) and uppercase (rows) do not differ from each other by the Scott–Knott test ($p \leq 0.05$).
Means followed by (*) do not differ from freshly harvested coffee using Student's t test.
SIAF: self-induced anaerobic fermentation

Table 3. Seed viability according to a tetrazolium test at sampling times 0 and 480 h of postharvest processing for the natural and pulping coffee.

Time (h)	Treatments	Viability (%)	
		Natural	Pulped
0	Freshly harvested	87.5*	85*
480	*S. cerevisiae* (SIAF)	52.5 bA	60.0 aA
	C. parapsilosis (SIAF)	67.5 bA	60.0 aA*
	T. delbrueckii (SIAF)	67.5 bA	70.0 aA*
	Control (SIAF)	65.0 bA	70.0 aA*
	Conventional process	90.0 aA*	60.0 aB*

Means followed by the same lowercase letter (columns) and uppercase (rows) do not differ from each other by the Scott–Knott test ($p \leq 0.088$). Means followed by (*) do not differ from freshly harvested coffee using Student's t test.
SIAF: self-induced anaerobic fermentation

Table 4. Electrical conductivity values ($\mu S.cm^{-1}.g^{-1}$) for coffee beans before fermentation (0 h) and at the end of drying (480 h) of natural and pulped coffee.

Time (h)	Treatments	Electrical conductivity ($\mu S.cm^{-1}.g^{-1}$)	
		Natural coffee	Pulped coffee
0	Freshly harvested	256.03*	206.50*
480	*S. cerevisiae* (SIAF)	398.35 bA	563.35 cB
	C. parapsilosis (SIAF)	381.85 bA	417.70 bA
	T. delbrueckii (SIAF)	376.80 bA	462.10 bB
	Control (SIAF)	309.68 aA	417.10 bB
	Conventional process	259.53 Ab*	209.00 Aa*

Means followed by the same lowercase letter (columns) and uppercase (rows) do not differ from each other by the Scott–Knott test ($p \leq 0.05$). Means followed by (*) do not differ from freshly harvested coffee using Student's t test.
SIAF: self-induced anaerobic fermentation

Supporting Information - Supplementary material

Table S.1 Specific primers used for qPCR analysis.

	Primers	
	Name	Sequence
S. cerevisiae[1]	SC-5fw	5′-AGGAGTGCGGTTCTTTCTAAAG-3′
	SC-3bw	5′-TGAAATGCGAGATTCCCCCA-3′
C. parapsilosis[2]	SADH-F	5´-GCTGCGGCTTCAACTGATGC-3´
	SADH-R	5´-CTTGGTCACGAGCCTCC-3´
T. delbrueckii[3]	L2	5′-CAAAGTCATCCAAGCCAGC-3´
	R2	5′-TTCTCAAACAATCATGTTTGGTAG-3′

Primers were described by (Díaz et al., 2013; Hays et al., 2011; Zott et al., 2010).

Table S.2 Volatile compounds found in green and roasted coffee samples processed by the natural method and pulped by self-induced anaerobic fermentation (SIAF), with and without yeast inoculation (*S. cerevisiae, T. delbrueckii, C. parapsilosis*, control SIAF) and conventional (without inoculation and SIAF).

Pulped roasted coffee					**Areas**		
Compounds	**Ret. Time**	**Ret. Index**	**Cont SIAF**	***S. cerevisiae***	***T. delbrueckii***	***C. parapsilosis***	**Conventional**
Acids							
Butanoic acid, 4-hydroxy-	10.958	1684	277627	173065	216956	257404	106014
n-Hexadecanoic acid	23.170	2866	28733	37354	31723	20712	22932
Octanoic acid	15.995	2122	28605	33619	28423	19687	19574
n-Decanoic acid	17.990	2263	14362	19531	15657	11289	9994
Alcohols							
Benzyl alcohol	14.044	1878	4110	3712	6432	7466	2363
Phenylethyl Alcohol	14.462	1939	11152	6853	17206	14109	11511
n-Nonadecanol	18.932	2373	1238	1023	789	1141	1264
Aldehydes							
Pentadecanal-	15.725	2108	5481	6577	7395	6936	4390
Esters							
Hexadecanoic acid, methyl ester	17.463	2201	15628	10660	10922	13086	8869
9,12-Octadecadienoic acid, ethyl ester	20.104	2509	9789	7948	6675	9450	3130
Furans							
Ethanone, 1-(2-furanyl)-	9.003	1491	275224	128109	193034	212150	186122
2-Furanmethanol, acetate	9.416	1531	276213	194537	216855	324868	126042
2-Furancarboxaldehyde, 5-methyl-	10.137	1603	1122037	716689	837276	1051208	930459
2-Furanmethanol	11.356	1710	3899658	2765713	3618695	4434811	2887350
5-Hydroxymethylfurfural	20.053	2503	51580	29695	37787	36196	44669
Furfural	8.201	1411	1643555	1088891	1811766	1506291	1835631
Ketones							
2-Pentadecanone	15.662	2104	5788	5368	5885	6258	5817
2-Heptadecanone	17.669	2226	6528	5183	5629	8311	5100

2-Pentadecanone, 6,10,14-trimethyl-	16.691	2159	10248	5961	8386	10819	9308
Lactones							
2(5H)-Furanone	12.476	1757	53349	34908	45398	50133	33731
4-Methyl-5H-furan-2-one	14.256	1909	21182	15129	16041	22857	14245
Others							
Caffeine	27.568	3379	1962909	996050	1011826	1167252	1488449
Phenols							
4-Hydroxy-2-methylacetophenone	17.218	2188	62541	47158	56063	61564	39231
Pyrans							
Maltol	15.215	2047	43159	76965	35035	45346	24798
Pyrazines							
Pyrazine, 3-ethyl-2,5-dimethyl-	8.568	1447	67170	43561	88360	117522	80596
3-Methyl-2-pyrazinylmethanol	14.591	1958	7267	4573	6802	9770	5388
Pyrazine, 2,6-dimethyl-	6.200	1213	432166	282628	264344	339502	239266
Pyridines							
3-Pyridinol, 2-methyl-	18.500	2322	23169	15635	15397	24597	20731
3-Pyridinol	19.555	2445	65825	58157	35452	45363	37321
Pyrroles							
1H-Pyrrole, 1-(2-furanylmethyl)-	13.290	1791	103168	60190	78674	111532	77777
Ethanone, 1-(1H-pyrrol-2-yl)-	15.064	2026	84843	57551	72660	105296	59527
1H-Pyrrole-2-carboxaldehyde	15.540	2095	82783	59308	65664	83985	52275
Indole	19.371	2424	26794	15906	24136	25051	20272
2-Pyrrolidinone, 1-butyl-	18.170	2284	30323	14493	16951	23817	7211
1H-Pyrrole-2-carboxaldehyde, 1-methyl-	16.498	2149	25740	18244	19775	25855	21302
Thiophenes							
2-Thiophenemethanol	14.719	1976	13882	9810	12173	16214	9151

Pulped green coffee				**Areas**			
Compounds	**Ret. Time**	**Ret. Index**	**Cont SIAF**	***S. cerevisiae***	***T. delbrueckii***	***C. parapsilosis***	**Conventional**
Acids							

n-Hexadecanoic acid	23.170	2866	4700	0	7321	5154	2386
Alcohols							
Benzyl alcohol	14.044	1878	12005	10969	12139	9996	4075
Phenylethyl Alcohol	14.462	1939	21695	13653	33599	15221	16050
n-Nonadecanol	18.932	2373	1712	914	1064	746	636
Aldehydes							
Pentadecanal-	15.725	2108	16985	11471	11355	12624	11534
Hexadecanal	16.742	2162	1950	2456	2231	2057	914
Esters							
Hexadecanoic acid, methyl ester	17.463	2201	5891	4714	4371	4840	2452
9,12-Octadecadienoic acid, ethyl ester	20.104	2509	5156	6441	5716	7295	2000
Hexanoic acid, 2-methylbutyl ester	13.130	1784	9535	0	0	7671	6753
Ketones							
2-Pentadecanone, 6,10,14-trimethyl-	16.691	2159	15479	10040	12459	11746	9943
2-Pentadecanone	15.662	2104	6260	5422	5181	5750	4344
2-Heptadecanone	17.669	2226	4485	4365	5218	4111	3737
Others							
Caffeine	27.568	3379	434400	380455	411042	439384	774363

Natural roasted coffee				**Areas**			
Compounds	**Ret. Time**	**Ret. index**	**Cont SIAF**	***S. cerevisiae***	***T. delbrueckii***	***C. parapsilosis***	**Conventional**
Acids							
Butanoic acid, 4-hydroxy-	10.958	1684	290221	272821	251229	259168	111848
n-Hexadecanoic acid	23.170	2866	11875	20933	11730	16765	21362
Octanoic acid	15.995	2122	12744	32100	21485	19440	21828
n-Decanoic acid	17.990	2263	0	7722	10688	6910	6676
Alcohols							
Benzyl alcohol	14.044	1878	18026	20433	17594	24494	6327
Phenylethyl Alcohol	14.462	1939	20729	27899	24604	18803	14390
n-Nonadecanol	18.932	2373	1181	1220	841	837	1101

Aldehydes							
Pentadecanal-	15.725	2108	7116	6053	6756	6630	4334
Esters							
Hexadecanoic acid, methyl ester	17.463	2201	14128	19364	25682	15553	8762
9,12-Octadecadienoic acid, ethyl ester	20.104	2509	10446	15212	16866	16768	7299
Ethyl Oleate	19.719	2464	1898	3123	3594	3488	1660
Octadecanoic acid, ethyl ester	19.575	2448	3213	5182	6274	6291	2349
Furans							
Ethanone, 1-(2-furanyl)-	9.003	1491	201262	190106	184218	192470	239237
2-Furanmethanol, acetate	9.416	1531	247051	290291	254090	334894	143570
2-Furancarboxaldehyde, 5-methyl-	10.137	1603	986204	871106	826672	872874	1056045
2-Furanmethanol	11.356	1710	3908118	3920841	3380060	4087136	3499688
5-Hydroxymethylfurfural	20.053	2503	26195	29042	23832	15611	42587
Furfural	8.201	1411	1530558	1133795	1120801	1510427	1904911
Ketones							
2-Pentadecanone, 6,10,14-trimethyl-	16.691	2159	9222	8173	6472	6665	7802
2-Pentadecanone	15.662	2104	4713	5301	10784	5790	3977
2-Heptadecanone	17.669	2226	3641	4938	6212	5274	3556
Lactones							
2(5H)-Furanone	12.476	1757	35501	35150	33172	32609	39291
4-Methyl-5H-furan-2-one	14.256	1909	19465	19879	16873	20228	16953
Others							
Caffeine	27.568	3379	1174155	951275	852242	679687	1130405
Phenols							
4-Hydroxy-2-methylacetophenone	17.218	2188	39155	26748	26727	32960	41630
Pyrans							
Maltol	15.215	2047	16679	22755	24323	21691	27770
Pyrazines							
Pyrazine, 3-ethyl-2,5-dimethyl-	8.568	1447	109718	110550	93618	120088	78328
3-Methyl-2-pyrazinylmethanol	14.591	1958	5905	7826	7420	6185	7324
Pyrazine, 2,6-dimethyl-	6.200	1213	401746	439554	328171	367961	404322

Pyridines							
3-Pyridinol, 2-methyl-	18.500	2322	10556	11541	13266	10210	15644
3-Pyridinol	19.555	2445	18085	22865	17959	13453	29755
Pyrroles							
1H-Pyrrole, 1-(2-furanylmethyl)-	13.290	1791	93546	82099	78910	86864	78449
Ethanone, 1-(1H-pyrrol-2-yl)-	15.064	2026	75055	73208	66446	69745	68248
1H-Pyrrole-2-carboxaldehyde	15.540	2095	56759	57931	58159	58129	57386
Indole	19.371	2424	24094	12155	17358	19120	20732
2-Pyrrolidinone, 1-butyl-	18.170	2284	31117	21452	18822	19277	8514
1H-Pyrrole-2-carboxaldehyde, 1-methyl-	16.498	2149	18629	18476	16467	15977	18280
Thiophenes							
2-Thiophenemethanol	14.719	1976	14138	13716	12184	12524	12672

Natural green coffee				**Areas**			
Compounds	**Ret. Time**	**Ret. Index**	**Cont SIAF**	***S. cerevisiae***	***T. delbrueckii***	***C. parapsilosis***	**Conventional**
Alcohols							
Benzyl alcohol	14.044	1878	27817	21723	23990	29494	16237
Phenylethyl Alcohol	14.462	1939	28427	26488	29974	21932	28729
n-Nonadecanol	18.932	2373	7762	0	0	1214	1102
Aldehydes							
Pentadecanal-	15.725	2108	15957	13294	17344	18579	22828
Hexadecanal	16.742	2162	4189	2859	3546	4562	2015
Esters							
Hexadecanoic acid, methyl ester	17.463	2201	6944	8297	9556	11374	4286
9,12-Octadecadienoic acid, ethyl ester	20.104	2509	5742	10218	13293	20492	3862
Ethyl Oleate	19.719	2464	983	2250	3767	3124	422
Octadecanoic acid, ethyl ester	19.575	2448	0	3332	4879	6141	1318
Hexanoic acid, 2-methylbutyl ester	13.130	1784	13021	14304	17013	15832	13048
Ketones							
2-Pentadecanone, 6,10,14-trimethyl-	16.691	2159	8802	9870	13675	11994	15887

2-Pentadecanone		15.662	2104	10242	9159	8764	8621	6041
2-Heptadecanone		17.669	2226	6168	6082	6790	5544	4154
	Others							
Caffeine		27.568	3379	237682	254989	263693	358079	318002

ARTIGO 2: Self-induced anaerobiosis fermentation in coffees inoculated with yeasts: Decreased viability of the seed and its relationship with the sensory quality of the beverage.

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Abstract

During coffee fermentation, metabolic germination processes are initiated causing changes in the chemical composition and quality of the coffee. Our objective was to evaluate the impact of coffee fermentation on the viability of the seed and the relationship of this impact with the chemical composition and sensory quality of the beverage with the use of yeasts as starter cultures (*Saccharomyces cerevisiae* (CCMA 0543), *Candida parapsilosis* (CCMA 0544) and *Torulospora delbrueckii* (CCMA 0684)), by Self-Induced Anaerobic Fermentation (SIAF) in natural and pulped coffee. The yeasts *S. cerevisiae* and *T. delbrueckii* presented the highest populations at the end of the process (>7 $\log_{10}$ cell/g) compared to *C. parapsilosis* (5.4 $\log_{10}$ cell/g), SIAF control (<7 $\log_{10}$ cell/g) and conventional process (<2.4 $\log_{10}$ cell/g) in natural and pulped coffee. A decrease in sugars (sucrose, glucose, and fructose) and acids (citric, malic, and succinic) was observed. The treatments processed by SIAF showed a statistically significant increase ($p \leq 0.05$) in the concentration of lactic acid at 180 hrs, compared to the concentration at 0 hrs. The enzymatic activity of catalase, alcohol dehydrogenase, esterase, endo-β-mannanase and isocitrate lyase showed the metabolic activity of germination during fermentation. Seed germination was decreased by fermentation and processing method. In general, during the SIAF with yeast inoculation, the embryo dies, causing the seed maintenance processes to stop, increasing the damage to the protective membranes and their permeability, allowing the diffusion of compounds produced during fermentation, enhancing flavor attributes, and improving the sensory quality of the beverage.

Keywords: Seed viability, Special coffee, Enzymatic activity, Microbial metabolisms, Sensory attributes, Coffee chemistry.

1. Introduction

In post-harvest coffee processing, the spontaneous fermentation is carried out by epiphytic microorganisms present in the coffee and those microorganisms from the environment (Silva et al., 2013). This fermentation can be to improvements in the chemical and sensory qualities of coffee, attributed to the modification by microbial metabolites produced in the fermentation that reaches the interior of the beans (Elhalis et al., 2020; Hadj Salem et al., 2020; Lee et al., 2015).

In coffee fermentation using starter cultures improve the control of the process and optimization of the fermentation time. Regardless of the type of processing (natural, pulped, and wet coffee), the self-induced anaerobiosis fermentation (SIAF) method have been shown to enhance the quality of the beverage (da Mota et al., 2022; Pereira et al., 2022; Schwan et al., 2023). SIAF is a fermentation method in which, the anaerobiosis is gradually produced by microbial metabolism that uses the remaining O_2 for its metabolic reactions, releasing CO_2 and volatile and non-volatile compounds (Cassimiro et al., 2023). Furthermore, this method positively impacts the fermentative performance of lactic acid bacteria (LAB) and yeasts during coffee processing (da Mota et al., 2020; Pereira et al., 2022).

During postharvest processing of coffee, the seed germination metabolism is initiated by enzymatic processes, resulting in nutrients consumption, and cell wall deterioration. This process affects the viability of the seed, modify the chemical composition of the coffee, and the sensory quality of the beverage (Coelho et al., 2015; Jimenez et al., 2023; Selmar et al., 2015; Taveira et al., 2015).

Therefore, the investigation of the enzymes involved (isocitrate lyase and endo-β-mannanase) in the germination process helps us better understand when germination metabolic processes are activated or stopped. For example, one of the critical enzymes activated in germinative metabolism is isocitrate lyase, a key enzyme in the glyoxylate cycle that determines

the embryogenesis transition, an indicator of cell division (Selmar et al., 2006; Taiz et al., 2017a).

Moreover, the presence of endo-β-mannanase indicate the development of the embryo since this enzyme is involved in the degradation processes of mannans (structural and storage polysaccharides in the endosperm cell wall) into monosaccharides and polysaccharides that are metabolized by the embryo growing (Campos-Vega et al., 2015; Dhawan, 2021). As well as catalase and esterase that indicate environmental stress and high concentrations may indicate a decrease in the maintenance processes or death of the embryo (Christy Santos et al., 2021).

Thus, this study aimed to evaluate the viability of coffee processed by SIAF fermentation inoculated with yeasts (*Saccharomyces cerevisiae* (CCMA 0543), *Candida parapsilosis* (CCMA 0544) and *Torulospora delbrueckii* (CCMA 0684)) and its relationship with the chemical composition of coffee.

2. Materials and Methods

2.1. Starter cultures

S. cerevisiae (CCMA0543), *C. parapsilosis* (CCMA0544), and *T. delbrueckii* (CCMA0684) were used as starter cultures in coffee processing by the SIAF method. These strains belong to the Collection of Cultures of Agricultural Microbiology (CCMA, Federal University of Lavras, Lavras, Minas Gerais, Brazil, and were isolated and identified from fermentative processes in coffee (Silva et al., 2013). Yeasts stored at - 80°C were reactivated and multiplied according to Martins et al. (2022), then they were centrifuged (3200 × g; 10 min) and resuspended in sterile water (500 mL) and inoculated into coffee at a concentration final of 10^7 cell/g of coffee. All the treatments were carried out in triplicate.

2.2. Coffee fermentation processing

The experiment was performed in the Fermentation Laboratory of the Department of Biology of the Federal University of Lavras. Coffee fruits *(Coffea arabica* L.) variety 'Topazio Amarelo' was harvested at the Monte Alegre Coffees farm in Alfenas, Minas Gerais, Brazil (S 1°59'56", W 54°4'58") at 850 m above sea level, for natural and pulped processing (Batista da Mota et al., 2022).

Forty liters of coffee were used for each cylindrical bioreactor (50L). The natural and pulped coffee was fermented using the SIAF method with inoculation (*S. cerevisiae* (CCMA0543*), C. parapsilosis* (CCMA0544), and *T. delbrueckii* (CCMA0684)), a control without inoculation (SIAF Control) and control by conventional processing (the traditional process where coffee is harvested and then dried in the sun on cement platforms). A total of 180 hrs was necessary to ferment the coffee (time defined with the monitoring of the temperature inside the bioreactor demonstrated by Jimenez et al. (2023). Samples (200 g) were taken at the beginning (0 hrs), middle (96 hrs), and end of the fermentation (180 hrs). After fermentation, the coffee was transferred to suspended terraces to dry in the sun until it reached 11–12% moisture.

2.3. Quantification of inoculated yeast populations

The inoculated yeast (*S. cerevisiae* (CCMA0543), *C. parapsilosis* (CCMA0544), and *T. delbrueckii* (CCMA0684)) was quantified during coffee fermentation (0, 96, and 180 hrs) in each sample, by qPCR using a QIAamp DNA Mini kit (Qiagen, Hilden, Germany). Each yeast was grown on YEPG agar at 28 °C for 24 hrs, followed by serial dilutions (1:10) from 10^8 to 10^3 cell/mL, with measurements at each point of the standard curve for use in qPCR (Batista et al., 2015), with the help of specific primers for each yeast (described in Jimenez et al. (2023)). The experiment was carried out in triplicate.

2.4. Analysis of chemical compounds by high-performance liquid chromatography (HPLC)

2.4.1. Carbohydrates and organic acids

Ten grams of coffee were mixed twice in 10 mL of Milli-Q water for 5 minutes by vortex. The solution extracted was centrifuged at 12,745 × g for 10 minutes at 4 °C. Acids extraction was necessary to adjust the pH to 2.11 by adding perchloric acid solution (16 mM). The supernatants were filtered through a 0.22 µm cellulose acetate membrane. Samples were stored at -18 °C for analysis.

Samples of natural and pulped coffee were analyzed at the 0, 96, and 180 hrs of fermentation using a high-performance liquid chromatography (HPLC) system (Shimadzu Corp., Japan). The analysis of carbohydrates (glucose, sucrose, and fructose) was performed according to da Mota et al. (2020), and organic acids (acetic, citric, lactic, malic, oxalic, succinic, and tartaric) were analyzed following the operating conditions described by (Evangelista et al., 2014). The carbohydrates and acids were identified by comparison with the retention times of authentic standards. Calibration curves were constructed with standards to quantify the chemical compounds.

2.5. Expression analysis of enzymatic activity

2.5.1. Catalase (CAT), Alcohol Dehydrogenase (ADH), Esterase (EST), Isocitrato Lyase (ICL)

Coffee seeds were ground with liquid nitrogen and Polyvinylpyrrolidone (PVP) 2% in a stainless-steel mill (IKA basic model A11, IKA, Germany), and subsequently, the samples were stored at -80 °C. The expression analysis of the enzymes catalase (CAT), alcohol dehydrogenase (ADH), esterase (EST), and isocitrate lyase (ICL) was carried out following the methodology described by Santos et al. (2021) with modifications. The enzymes were extracted with 0.2 M Tris HCl buffer pH 8.0 + (0.2% 2-Mercaptoethanol) + 0.4% PVP + 0.4% Poly Ethylene Glycol (PEG) 6000 + 1 mM Disodium Salt Dihydrate (EDTA). In addition, the

isocitrate lyase extraction was added 0.1% de Phenylhydrazine. The buffer was used in the proportion of 320 µl per 100 mg of seeds.

The extract was shaken with a vortex and left to rest at 4 °C for 60 minutes, then centrifuged at 12,000 rpm for 60 minutes at 4 °C. An electrophoretic run was performed on a two-phase polyacrylamide gel system: separation gel (7.5%) and concentration gel (4.5%). The gel/electrode system used was Tris-glycine pH 8.9. The supernatant (60 µl) was applied to the gel. The electrophoretic run was carried out at 150 V for 6 hrs (Christy Santos et al., 2021). The gels were revealed, according to Alfenas (2006).

2.2. Endo-β-mannanase

The endo-β-mannanase enzyme activity was performed for each treatment at different times of the fermentation process (0, 96, 180 hrs for the SIAF treatments and 0 and 480 hrs for the conventional process), following the methodology described by (de Freitas et al., 2017).

2.6. Germination test

Coffee samples from times 0 and 180 hrs were used with four replicates of 50 seeds without parchment (extracted by hand) were used for each treatment and processing method. Sowing was carried out on "germitest" type paper towels (paper rolls), moistened with distilled water in the amount of 2.5 times the mass of dry paper, and placed to germinate at 30 ºC in the presence of light (Abreu et al., 2017). The final count was carried out 30 days after sowing, computing the normal seedlings in each repetition according to the criteria established by the Standards for Seed Analysis (Brasil, 2009).

2.7. Sensory analyses

The coffees were performed with a panel of five trained coffee tasters with Q-grade coffee

certificates following Specialty Coffee Association (SCA) protocol (SCA, 2018). The attributes: fragrance, aroma, flavor, aftertaste, acidity, body, uniformity, balance, sweetness, cleaning, defects, and general score were evaluated. Sensory analysis was performed after approval by the Ethics Committee of the Federal University of Lavras (CAAE: 63924722.9.0000.5148).

2.8. Statistics analyses

The experiment was carried out with a completely randomized design (DIC) with a 4x3 factorial arrangement (with four treatments being the three yeasts and a control x time 0, 96, and 180 hrs), in addition, a comparison of means was carried out in the conventional process for two times (0 and 480 hrs), to evaluate organic acids and sugars. In the same way, the population behavior of the yeasts was monitored, comparing each inoculated treatment with the SIAF control using a 2x3 mixed factorial design (yeast and SIAF control x time: 0, 96, and 180 hrs) and comparison of means for the processes conventional for two times (0 and 480 hrs) for each yeast. In addition, for the germination test, a 5x2 factorial arrangement was used (3 yeasts, SIAF control, and conventional process x time: 0 and 180 hrs), and for the activity of the endo-β-mannanase enzyme, a 10 x 2 comparison of means was made. (10 treatments x 2 processing methods).

Analysis of variance (ANOVA) was used using the Tukey test ($p \leq 0.05$) for sugars, acids, and yeast populations. In addition, for the final score of the sensory evaluation, the Fisher LSD test ($p \leq 0.05$) and the Scott–Knott test ($p \leq 0.05$) were used for the enzymatic activity of endo-β-mannanase. The Sisvar software version 5.6 (Ferreira, 2014) was used for the statistical analysis.

3. Results

3.1. Quantification of inoculated yeast populations

Saccharomyces cerevisiae, *Candida parapsilosis*, and *Torulaspora delbrueckii* are commonly found during the coffee fermentation process, and their population was monitored using the qPCR technique. When analyzing the SIAF control and the conventional process at the beginning of the process (0 hrs), the yeasts with the highest populations were *T. delbrueckii* (6.07 $\log_{10}$ cell/g) and *S. cerevisiae* (5.04 $\log_{10}$ cell/g) for natural coffee and the yeasts *C. parapsilosis* (2.68 $\log_{10}$ cell/g) and *T. delbrueckii* (2.67 $\log_{10}$ cell/g) for pulped coffee.

Additionally, it was observed that the populations of the *C. parapsilosis* starter culture decreased during fermentation (6.28 to 5.4 $\log_{10}$ cell/g in natural coffee and 5.73 to 5.39 $\log_{10}$ cell/g in pulped coffee); a similar behavior was observed in the SIAF controls for this yeast (3.23 to 2.85 $\log_{10}$ cell/g in natural coffee and 2.68 to 2.34 $\log_{10}$ cell/g in pulped coffee) (Figure 1.). In the conventional process, the populations of *S. cerevisiae* and *T. delbrueckii* yeasts decreased at 480 hrs compared to 0 hrs in both processing methods (natural and pulped coffee). The population of the yeast *C. parapsilosis* decreased in natural coffee but not in pulped coffee, where it presented a statistically significant population increase ($p \leq 0.05$) (2.68 $\log_{10}$ cell/g at 0 hrs and 2.92 $\log_{10}$ cell/g at 480 hrs) (Figure 1.).

3.2. Carbohydrates

The concentration of sugars (sucrose, glucose, and fructose) differed significantly ($p \leq 0.05$) in the fermentation time (Table 1.). In natural coffee, the highest concentrations of fructose (8.13 g/kg), glucose (6.41 g/kg), and sucrose (1.7 g/kg) were for *S. cerevisiae*, *T. delbrueckii* and *C. parapsilosis* and conventional process at 0 hrs. However, the SIAF control presented higher concentrations of glucose (9.71 g/kg) and fructose (11.12 g/kg) at 96 hrs. At 180 hrs, the lowest concentrations were observed for all sugars.

In pulped coffee, the highest concentration was observed for sucrose (2.19 g/kg), glucose

(0.41 g/kg) in 0 hrs. At 180 hrs, a decrease statistically significant differences ($p \leq 0.05$) in sucrose were observed in all fermentations. Glucose presented a decrease for *C. parapsilosis* (0.41 a 0.04 g/kg), SIAF control (0.41 a 0.14 g/kg), conventional process (0.41 a 0.12 g/kg) and no statistically significant differences ($p \leq 0.05$) for *S. cerevisiae* and *T. delbrueckii*. For fructose, absent at 0 hrs (0.00 g/kg), was detected in concentrations*; S. cerevisiae* (0.36 g/kg), *C. parapsilosis* (0.38 g/kg), *T. delbrueckii* (0.42 g/kg), SIAF control (0.17 g/kg) at 180 hrs and for the conventional process (0.59 g/kg) at 480 hrs.

3.3. Organic acids

Acids (citric, malic, succinic, and lactic) were detected during coffee fermentation (Table 2.). Citric acid presented the highest concentration at 0 hrs of fermentation (8.85 g/kg in natural coffee and 3.01 g/kg in pulped coffee). In natural coffee, a significant decrease ($p>0.5$) was observed in the concentration of this acid after 96 hours in *S. cerevisiae* (4.85 to 1.93 g/kg) and *T. delbrueckii* (4.85 to 2.20 g/kg) with a final concentration at 180 hours of 1.67 g/kg for *S. cerevisiae* and 2.39 g/kg for *T. delbrueckii*. However, *C. parapsilosis*, SIAF control, and conventional process maintained the initial concentration (0 hrs) of this acid during fermentation without statistically significant differences ($p \leq 0.05$). The pulped coffees did not present alterations in the concentration of citric acid during the time (0-180 hrs).

Malic acid presented a decrease in concentration during the fermentation time (0-180 hrs) except for the conventional process at 480 hrs, which presented an increase in concentration (1.13 to 2.02 g/kg in natural coffee and 1.74 to 2.75 g/kg in pulped coffee).

Succinic acid did not present a statistically significant difference ($p>0.5$) during fermentation for *C. parapsilosis*, *T. delbrueckii*, and SIAF control in natural and pulped coffee. However, *S. cerevisiae* presented a statistically significant decrease ($p>0.5$) in natural coffee (0.76 to 0.42 g/kg) and pulped coffee (1.17 to 0.35 g/kg). In addition, the conventional process

increased at 480 hours in natural coffee (0.76 to 1.25 g/kg) and pulped coffee (1.17 to 2.22 g/kg).

Lactic acid was not detected at 0 hrs, and the production of this acid was observed starting at 96 hrs in SIAF coffees, obtaining the highest concentrations at 180 hrs. This acid was not detected in the conventional process (Table 2.).

3.4. Expression analysis of enzymatic activity

3.4.1. Catalase (CAT)

Figures 2. A1-A2 shows the enzymatic activity of catalase, present in the conventional process at 480 hrs with 11% moisture and with less activity in the SIAF control at 96 hrs with 57% moisture. The pulped coffees presented higher intensity in the enzymatic activity.

3.4.2. Alcohol Dehydrogenase (ADH)

Figures 2. B1-B2 shows the enzymatic activity of alcohol dehydrogenase (ADH). The fresh fruits (0 hrs) with 61% and 53% moisture showed enzymatic activity in natural and pulped coffee. In natural coffee at 96 hrs, enzymatic activity is observed in *S. cerevisiae* and *T. delbrueckii* to a lesser extent in SIAF control and absent in *C. parapsilosis*. At 180 hrs, enzymatic expression was observed in all coffees fermented by SIAF.

In pulped coffee at 96 hrs, enzymatic activity is observed in coffees fermented by SIAF. At 180 hrs, enzymatic activity was observed in all the coffees fermented by SIAF. However, this enzyme did not show activity for natural and pulped coffee in the conventional process at 480 hrs with 11% moisture.

3.4.3. Esterase (EST)

This enzyme always showed enzymatic activity; in fresh fruits (or hrs), at 96 hrs of

fermentation, and 180 hrs with less intensity. In addition, the conventional process at 480 hrs also shows the presence of this enzyme (Figures 2. C1-C2).

3.4.4. Isocitrato Lyase (ICL)

The activity of the ICL is shown in Figure 2. D1-D2, observing the higher activity of this enzyme at 96 hrs in both processing methods (natural and pulped coffees). In addition, a decrease in activity was observed at 180 hrs except for the treatment inoculated with *T. delbrueckii*, which presented ICL activity at this time for both processing methods (natural and pulped coffee). Conventional processing did not show ICL activity at 480 hrs.

3.4.5. Endo-β-mannanase

The endo-β-mannanase activity (Table 3) showed significant differences ($p \leq 0.05$) between the treatments and the processing methods. For natural coffee, it was observed that the freshly harvested fruit coffee presented the highest enzymatic activity when compared with the other treatments at different times. In contrast, in the pulped coffee, there was a more significant enzymatic variation, where the conventional process at 480 hrs presented the higher enzymatic activity followed by recently harvested coffee fruits; the other treatments presented variations during times 96 and 180 hrs in lower concentrations than recently harvested coffee fruits.

When comparing the enzymatic activity of endo-β-mannanase between the processing methods (natural and pulped), higher enzymatic activity was observed in the pulped coffees at times 0, 96 hrs for the coffees processed by SIAF and at 480 for the conventional process, without statistical differences at 180 hrs.

3.5. Germination test

The results of the germination test are shown in Table 4., with significant differences ($p \leq$

0.05) between treatments and between the times of freshly harvested coffee before (0 hrs) and at the end of fermentation (180 hrs). The fresh fruit presented the highest % germination (94% natural coffee and 93% pulped coffee); no significant statistical difference ($p \leq 0.05$) was observed for natural coffee by conventional processing (85%) at 180 hrs, compared with freshly harvested coffee.

In the natural coffee, a decrease in the % germination was observed in all treatments processed by SIAF, watching the lowest % in the treatments *S. cerevisiae* (63%) and *C. parapsilosis* (58%). In pulped coffee, all treatments, including conventional processing, showed a decrease in germination % at 180 hrs, with the *S. cerevisiae* treatment being the treatment with the lowest germination 42%.

3.6. Sensory evaluation

All coffees were classified as specialty coffees with scores above 80 (Figure 3). A statistically significant difference ($p \leq 0.05$) was observed for the treatments inoculated with the yeast *C. parapsilosis* with the highest sensory score (85.92) (described in Jimenez et al. (2023)), followed by the *yeast S. cerevisiae* (85.00) compared to the other treatments in coffees processed by natural coffee. The treatment inoculated with the yeast *T. delbrueckii* presented the highest score (86.50), statistically significant ($p \leq 0.05$) compared to the other treatments processed with pulped coffee (described in Jimenez et al. (2023)). In addition, the tasters described the presence of different sensory attributes, with differentiated flavors and aromas for each treatment (Figure 3.).

The sensory evaluation by tasters showed a greater variety of sensory descriptors in the SIAF-fermented coffees (Figure 3.), as well as a vinous fermentation flavor in the SIAF-fermented coffees, except for the SIAF control in pulped coffee, which did not present this sensory feature.

The conventional process presented a lower number of sensory descriptors compared to the coffees fermented by SIAF. The fruity, sweetened, floral, chocolate, spices, chestnut, and fermented flavors stand out among the inoculated treatments. Different sensory descriptors were detected in natural coffee, *S. cerevisiae* presented a flavor of tropical fruit liquor, and in natural coffee, the conventional process presented a tobacco flavor.

4. Discussion

SIAF is a new technology used in coffee fermentation to produce specialty coffees (da Mota et al., 2022); in this research, SIAF was used with yeasts as starter cultures. When analyzing the population behavior of yeasts during coffee fermentation, the yeasts *S. cerevisiae* and *T. delbrueckii* demonstrated to enhance their populations during fermentation using this fermentation method. The behavior of these yeasts may be related to the environmental conditions developed inside the bioreactor (oxygen concentration, temperature, aw, pH), which may favor or limit yeast growth, allowing the growth or inhibition of other yeasts microorganisms present in coffee (lactic acid bacteria, mesophiles, and fungi) (Evangelista et al., 2015; Martinez et al., 2017; Martins et al., 2019; Pereira et al., 2022). In addition, SIAF favors the interaction of microorganisms with the inoculum, allowing the creation of ideal conditions for yeast growth, such as decreased oxygen and reduced pH (da Mota et al., 2022; Pereira et al., 2022).

Furthermore, *S. cerevisiae* showed a favorable growth in the SIAF and may be related to, adaptive changes to stress, resulting in aerobic or anaerobic growth, depending on environmental conditions (Marks et al. 2008),

Candida parapsilosis presented a decrease in its population during the fermentation time, and this population behavior may be due to the influence of the communities of microorganisms present in the coffee (bacteria, yeasts, fungi) that compete for nutrients (Bressani et al., 2021).

In the SIAF control, the yeasts *S. cerevisiae*, and *T. delbrueckii* showed a population greater than 5 Log_{10} cell/g in natural coffee and with populations less than 3 Log_{10} cell/g in pulped coffee. These smaller populations in the pulped coffee are related with part of the population of epiphytic microorganisms and those microorganisms from the environment has been removed along with the husk. *Candida parapsilosis*, the SIAF control populations reached 3 Log_{10} cell/g in natural coffee and 2.68 Log_{10} cell/g in pulped coffee. The high populations reported by the yeasts *S. cerevisiae* and *T. delbrueckii* in the SIAF controls, may have inhibited the growth of *C. parapsilosis* by competing for nutrients (Cassimiro et al., 2022; Martinez et al., 2017; Silva et al., 2008). Considering the population growth of microorganisms present in coffee, sugars are considered the primary source of energy for these microorganisms (Kim et al., 2022) and essential in the coffee fermentation process due to their influence on the sensory characteristics of coffee, transforming them into other compounds responsible for the desired aromas and flavors. in the drink (Prakash et al., 2022).

Differences were observed in sugar concentrations at the beginning of coffee fermentation (Table 1), with lower concentrations in pulped coffees. Also, with the removal of the coffee husk, there is a loss of the compounds present in the fruit, reflected in the sugar concentrations, which can vary depending on the type of processing (natural or pulped) (da Mota et al., 2020; Knopp et al., 2006). At the end of the fermentation, the levels of sucrose, glucose, and fructose decreased concerning the initial concentrations, as a result of the growth and metabolism of the starters and the microorganisms present in the coffee, resulting in the production of non-volatile compounds and 40 compounds volatiles (described in Jimenez et al. (2023)) that allowed modification of the flavor profiles of coffee (Wang et al., 2020).

Citric, malic, and succinic acids are found naturally in coffee (Bressani et al., 2021); together with sugars are responsible for developing flavor and aroma during coffee roasting through the Maillard reaction and Stecker degradation (Fernandes Fernando, 2019). At the

beginning of the process (0 hrs), citric, malic, and succinic were identified. Citric acid was the primary acid found with concentrations of 4.85 g/kg in natural coffee and 3.01 g/kg in pulped coffee.

Almost a total decrease of malic acid was observed in the coffees processed by SIAF; at the same time, lactic acid production was observed. This behavior of the acids may be related to the lactic acid bacteria present in coffee fermentation, which use these acids as carbon sources in aerobic respiration processes through tricarboxylic acid (TCA), increasing or decreasing their concentration during coffee processing (Mendes Ferreira and Mendes-Faia, 2020).

This decrease in malic acid may be linked to the production of lactic acid through the metabolic pathways of LAB (lactic acid bacteria) (Mendes Ferreira and Mendes-Faia, 2020), naturally present in coffee (Avallone et al., 2001; Evangelista et al., 2015), which are favored in their growth by SIAF, where the oxygen concentration is low, producing lactic acid, favoring the increase in coffee acidity (Bressani et al., 2020).

In conventional processing, no variation in malic acid concentrations was observed at the beginning of the process, and lactic acid was not detected throughout the process. Results were influenced by the processing method used, subjected to drying immediately after harvest, and processed in an open environment (with oxygen) throughout the process, limiting the production of metabolites by LAB (Papadimitriou et al., 2016).

On the other hand, this study demonstrated that the coffee seed initiates the metabolic activity of germination processes during the fermentative processing of coffee (Figure 2) by monitoring key enzymes. endo-β-mannanase activity showed the weakening of mannans (polysaccharides) in the endosperm cell wall, allowing the initiation of endosperm radicle protrusion (seed germination initiation) (de Freitas et al., 2017). The activity of this enzyme is regulated by the presence of phytohormones, such as the abscisic acid naturally present in coffee, which acts by inhibiting the action of the enzymes; by removing the husk from the

coffee, the abscisic acid is lost, and the enzymatic activity of the endo-β-mannanase is released, inducing the degradation of the endosperm and, consequently, favors germination (Bewley and Black, 1994; da Silva et al., 2004), which was reflected in higher enzymatic activity in pulped coffees, with higher activity in the conventional process at 480 hrs (dry coffee with 11% humidity), related results. What was described by(de Freitas et al., 2017), mentions that this enzyme can present a more significant activity in seed deterioration processes.

Therefore, coffee germination decreased after fermentation by SIAF with and without yeast inoculation for natural and pulped coffee. The conventional process in natural coffee did not show a decrease in germination after coffee processing (85.00 %), but there was a decrease in germination in pulped coffee (67 %) (Table 4). Additionally, the decrease in germination in pulped coffee by conventional processing may be related to the removal of the seed shell, which functions as a protective barrier for the embryo, and its removal decreases viability, allowing the permeability of the embryo, accelerating degenerative processes (Taiz et al., 2017b, 2017c).

The decrease in seed germination by SIAF is related to the metabolic activity of active germination during fermentation, demonstrated by the enzymatic expression of ICL (Figure 2. D1-D2), which is an indicator of cell division within the seed (Bytof et al., 2007; Kramer et al., 2010). Furthermore, the activity of this enzyme increases during seed germination, obtaining maximum values when the maximum proportion of degraded lipids and sucrose synthesis occurs (Bewley and Black, 1994; Coelho et al., 2015).

Also, the consumption of nutrients by the fermentative processes of the microorganisms and by the metabolic processes of active germination during fermentation (Kim et al., 2022; Knopp et al., 2006) was observed, causing changes in the chemical composition of the coffee (Tables 1. and 2.). Knopp et al., (2006) report that simple sugars are mainly the compounds affected by the activation of germination metabolisms during coffee fermentation, which could show an existing relationship with the decrease in seed germination observed in Table 4.

Our results show the existence of anaerobiosis due to the enzymatic expression of ADH during fermentation (Figure 2. B1-B2). ADH activity is induced by stress conditions caused by anaerobic processes that accelerate seed deterioration (Carvalho et al., 2014; Moreno et al., 2019). Likewise, this anaerobiosis causes alternative metabolic pathways (anaerobic respiration) inside the seed (cellular maintenance activities, cellular respiration), which result in greater consumption of energy reserves, as well as stoppage of germination metabolic processes due to lack of suitable conditions (dormancy), until the death of the embryo (Bewley and Black, 1994; Švubová et al., 2021; Taiz et al., 2017b).

Besides, the expression of the CAT enzyme during fermentation shows mechanisms of prevention of oxidative damage involved in removing hydrogen peroxides (H_2O_2) (Baker et al., 2023). However, when analyzing the enzymatic activity of the CAT, the conventional process at 480 hrs with 11% humidity showed greater intensity in the gel; this may be related to the increase in the production of reactive oxygen species and stimulation of the generation of H_2O_2 during drying (Christy Santos et al., 2021).

Intense EST enzymatic activity was observed in conventional processing (Figure 2. C1-C2) for natural and pulped coffee. However, the expression of enzymes in electrophoresis gels can be influenced by the moisture of the seed. In the Control (0 hrs) treatment, beans with high moisture also showed high EST activity, associated with less damage to membrane systems (Coelho et al., 2015). Not in so much (Figueiredo et al., 2021) describe the EST enzyme as an indicator of seed deterioration, with an essential catalytic function in cell detoxification, and its higher expression may be associated with a more significant disruption of cell membranes that occurs during drying.

The enzymatic activity observed during coffee fermentation (CAT, ADH, EST, and ICL) (Figure 2.) is related to seed deterioration processes; in respiratory processes, the enzymatic activity of ADH, responses to oxidative processes related to the elimination of free radicals,

CAT, rupture of the membranes of EST seeds and those related to the degradation of reserve materials such as ICL (Coelho et al., 2015; Moreno et al., 2019).

The fermentation process with the use of bioreactors and starter helps to standardize the process, ensuring the domination of the process by a starter culture, which with the help of adequate monitoring, modifies or intensifies some sensory descriptors of the beverage (caramel, chocolate, malic acidity, citric acidity, fruit flavors, and aromas, spices, floral, sweetened) (Figure 3) produced by microbial metabolites that improve the sensory characteristics of coffee (Batista da Mota et al., 2022; Cassimiro et al., 2022). This improvement in sensory quality is directly related to changes in the chemical composition of the coffee caused by the fermentation metabolism of the microbiota present in the coffee, by processes of the germinative metabolism of the seed, as well as the protective enzymatic activity of the seed that produces metabolites that alter the chemical composition of the coffee seed, giving rise to differential aromatic compounds for each treatment (Braga et al., 2023; Hadj Salem et al., 2020; Hill and Borém, 2020).

All coffees were classified as specialty coffees (>80 points). The natural coffees presented a statistically significant difference ($p \leq 0.05$) between the treatments. The coffees inoculated with the yeast *T. delbrueckii* by pulped coffee obtained the highest sensory score (86.50) with a statistically significant difference ($p \leq 0.05$) among the other treatments. On the contrary, the coffees processed by the conventional process obtained the lowest scores; 83.75 in natural coffee and 84.25 in pulped coffee (Figure 3) (described in Jimenez et al. (2023)). However, it did present differences between the sensory attributes for each treatment. The *S. cerevisiae* presented a more significant number of sensory attributes perceived by the tasters with flavors of citrus, red, and raisins, sweetened with caramel, molasses, honey, vanilla, flavors of white chocolate, chestnuts, walnuts, and hazelnuts, as well as a flavor of vinous fermented, in *T. delbrueckii* the sweetened taste of sugar cane and nutmeg stands out with the presence of other

sensory attributes shown in Figure 3. The sensory scores did not show a relationship with the viability of the seed (% germination) since the conventional process in natural coffee presented the highest % germination (85.00) (Table 4.) without statistically significant difference ($p \leq 0.05$) with fresh coffee harvested (% germination of coffee after harvest). In contrast, the treatment inoculated with *C. parapsilosis* (higher sensory score) obtained the lowest % germination. Therefore, no relationship was observed between sensory quality and seed viability in coffees fermented with yeast inoculation by SIAF.

5. Conclusions

The activity of key enzymes of the germinative metabolism of the seed shows the beginning of the germinative process, as well as the degradation suffered by the embryo during coffee fermentation. There is a decrease in the viability of the coffee seed when it is processed by self-induced anaerobiosis fermentation (SIAF) in natural and pulped coffees. The reduction of the viability of the coffee seed does not have a direct relationship with the sensory quality of the coffee. The use of yeasts as starter cultures by SIAF, *Candida parapsilosis* (CCMA 0544), *Saccharomyces cerevisiae* (CCMA 0543), and *Torulaspora delbrueckii* (CCMA 0684) improve the production of specialty coffees with differentiated sensory attributes and can become starters that help control the coffee fermentation.

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Figure captions

Figure 1. Population behavior of *S. cerevisiae*, *T. delbrueckii*, and *C. parapsilosis* by qPCR during coffee fermentation (0, 96, and 180 hrs). Means followed by lowercase letters (treatments) and uppercase letters (time) equals do not differ from each other, using the Tukey test ($p \leq 0.05$). a= Natural coffee, b= Pulped coffee, SIAF= Self-induced anaerobiosis fermentation, Inoc = Inoculated, Cont = Control, Conv = Conventional process.

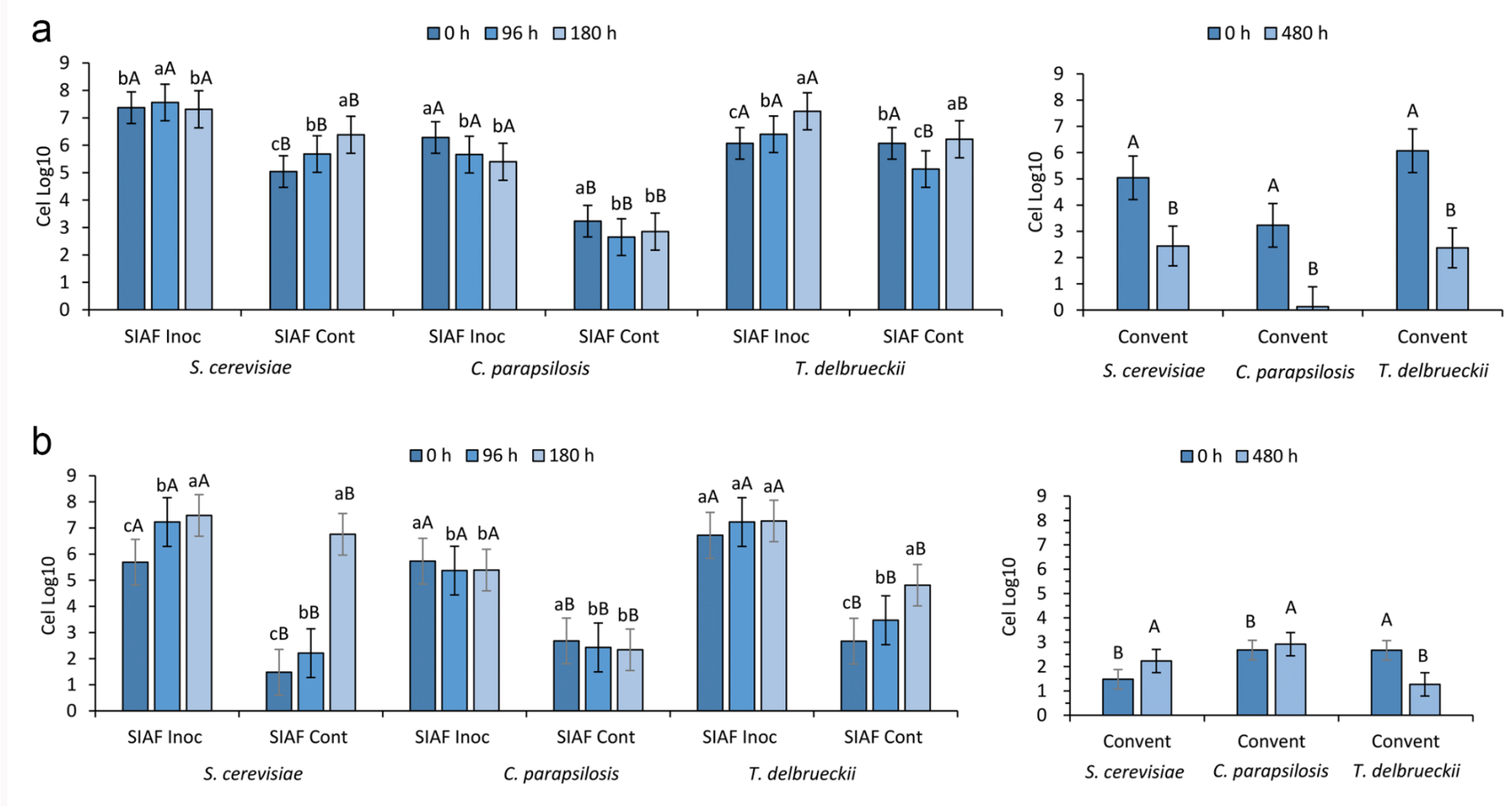

a
0 h
96 h
180 h
Cel Log10
SIAF Inoc
SIAF Cont
S. cerevisiae
C. parapsilosis
T. delbrueckii
0 h
480 h
Convent
b

Figure 2. Electrophoretic expression of enzymes

Capital letters symbolize the analyzed enzymes: **A** Catalase (CAT), **B** Alcohol dehydrogenase (ADH), **C** Esterase (EST), and **D** Isocitrate lyase (ICL).

The numbers (1 and 2) followed by the letters symbolize the processing method: 1= Natural coffee and 2= Pulped coffee.

The numbers from 1-10 symbolize the treatments during the time: 1= Conventional process (480 h), 2= Freshly harvested fruit (0h), 3= Control SIAF (96 h), 4= *S. cerevisiae* (96 h), 5= *T. delbrueckii* (96 h), 6= *C. parapsilosis* (96 h), 7= Control SIAF (180 h), 8= *S. cerevisiae* (180 h), 9= *T. delbrueckii* (180 h), 10 = *C. parapsilosis* (180 h).

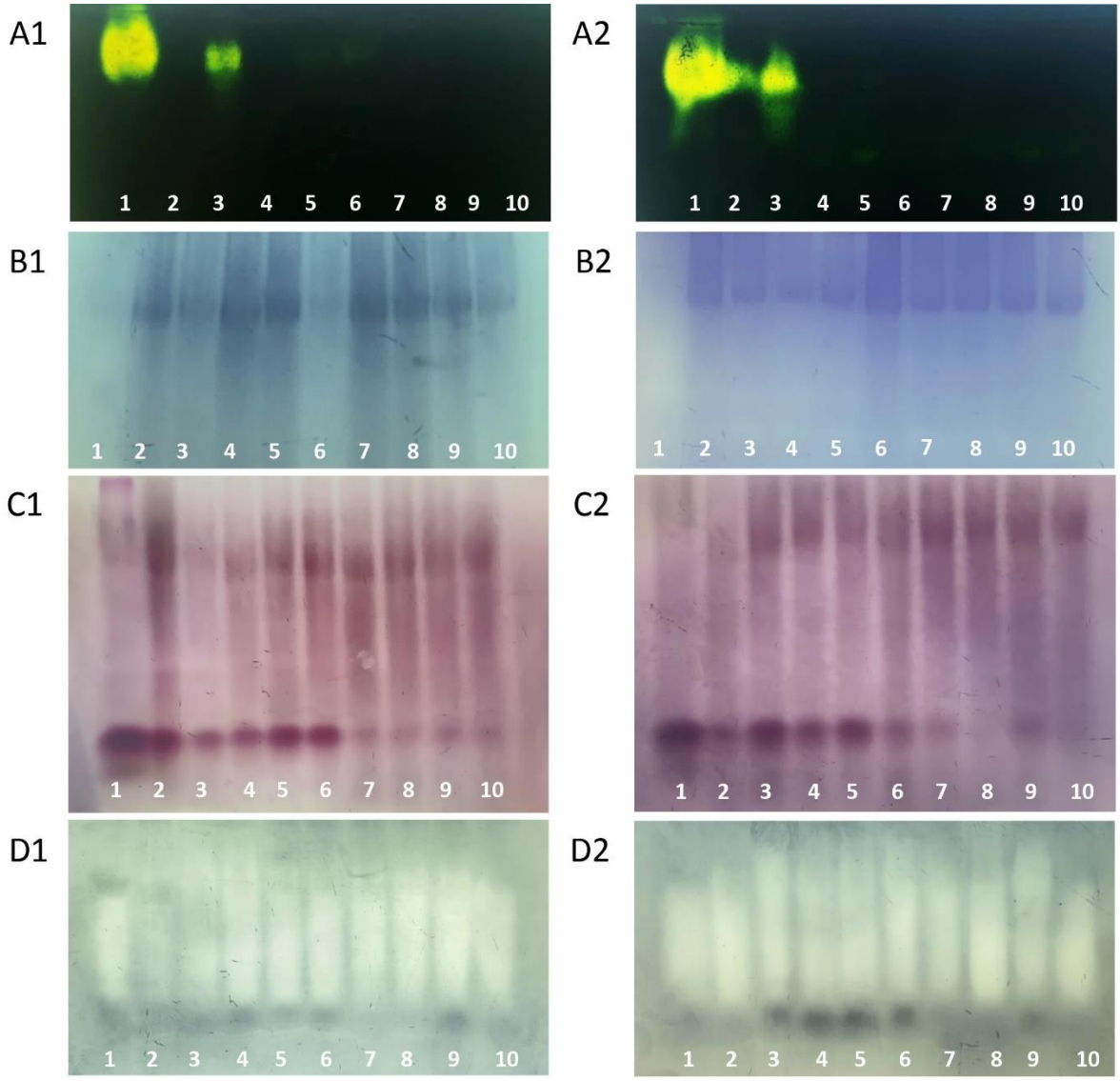
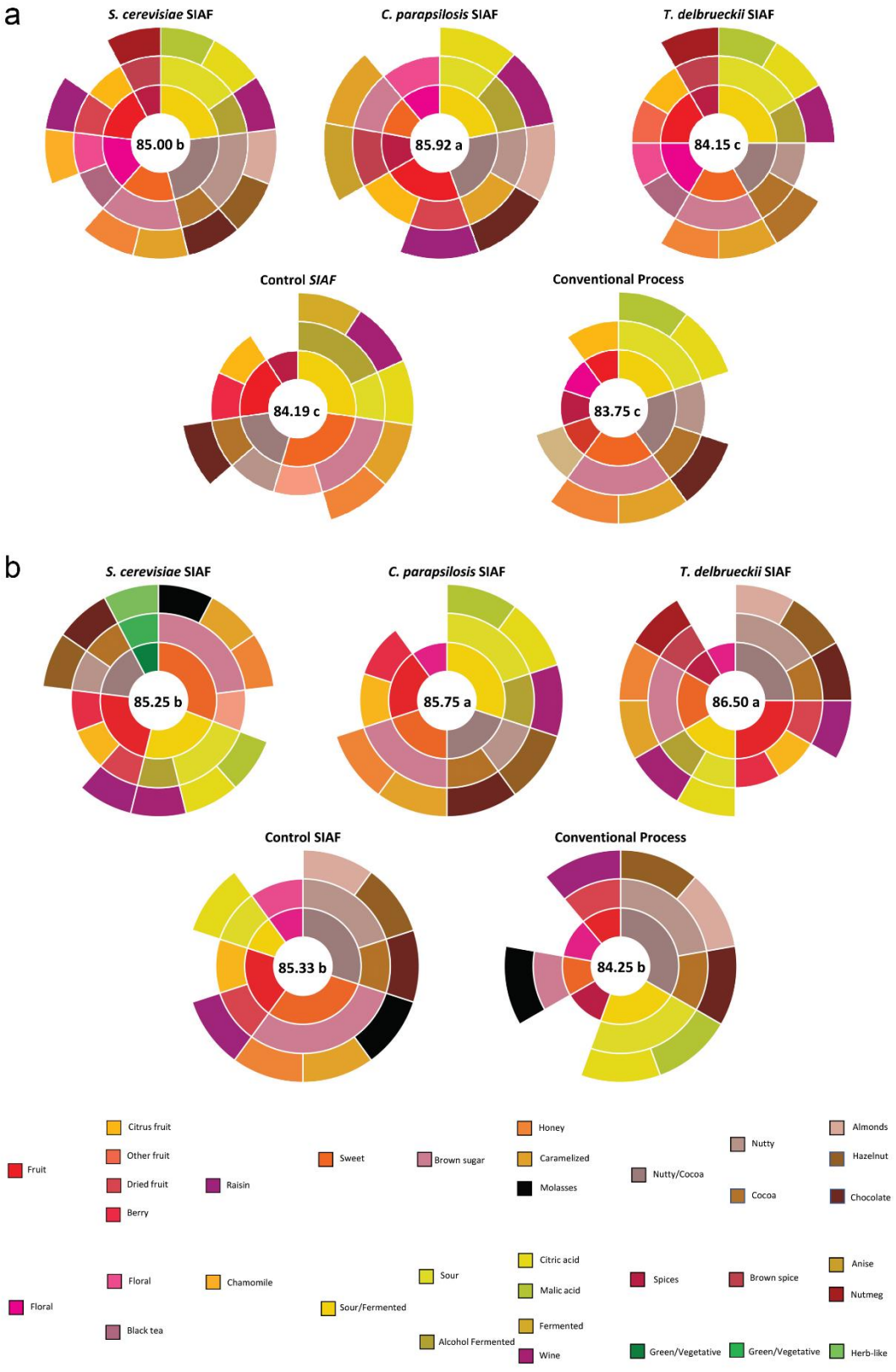


Figure 3. Sensory descriptors and the final score of the fermented coffee. Descriptor groupings follow the SCA flavor wheel. Different lowercase letters indicate a statistically significant difference ($p < 0.05$) by Fisher's LSD Test (For the interpretation of the references to color in the legend of this figure, the reader is referred to the web version of this article). a= Natural coffee and b= Pulped coffee.


a
S. cerevisiae SIAF
C. parapsilosis SIAF
T. delbrueckii SIAF
85.00 b
85.92 a
84.15 c
Control SIAF
Conventional Process
84.19 c
83.75 c
b
S. cerevisiae SIAF
C. parapsilosis SIAF
T. delbrueckii SIAF
85.25 b
85.75 a
86.50 a
Control SIAF
Conventional Process
85.33 b
84.25 b
Fruit
Citrus fruit
Other fruit
Dried fruit
Raisin
Berry
Sweet
Brown sugar
Honey
Caramelized
Molasses
Nutty/Cocoa
Nutty
Cocoa
Almonds
Hazelnut
Chocolate
Floral
Floral
Chamomile
Black tea
Sour
Sour/Fermented
Alcohol Fermented
Citric acid
Malic acid
Fermented
Wine
Spices
Brown spice
Anise
Nutmeg
Green/Vegetative
Green/Vegetative
Herb-like

Tables

Table 1. Sugars (g/kg) concentration in coffee before and at the end of processing by SIAF and conventional process in natural and pulped coffee.

Treatments	Time	Sucrose		Glucose		Fructose	
		Natural	Pulped	Natural	Pulped	Natural	Pulped
Freshly harvested fruits	0 h	1.70 *A	2.19 *A	6.41 *A	0.41 *A	8.13 *A	ND *B
S. cerevisiae SIAF	96 h	0.04 bB	0.35 aB	1.67 bB	ND aB	2.39 bB	0.31 aA
C. parapsilosis SIAF		0.08 bB	0.30 aB	2.20 bB	0.14 aB	3.61 bB	0.35 aA
T. delbrueckii SIAF		ND bB	0.20 aB	2.54 bB	ND aB	3.15 bB	ND bB
Cont SIAF		0.62 aB	0.36 aB	9.71 aA	ND aB	11.12 aA	ND bB
S. cerevisiae SIAF	180 h	ND aB	0.08 bB	0.68 aB	0.44 aA	0.98 aB	0.36 aA
C. parapsilosis SIAF		0.06 aB	0.60 aB	1.02 aB	0.04 bB	1.33 aB	0.38 aA
T. delbrueckii SIAF		ND aB	ND bB	1.11 aB	0.37 aA	1.53 aB	0.42 aA
Cont SIAF		ND aC	0.84 aB	1.68 aB	0.14 bB	3.35 aB	0.17 aA
*Conventional Process	0 h	1.70 A	2.19 A	6.41 A	0.41 A	8.13 A	ND A
	480 h	0.45 B	0.33 B	1.70 B	0.12 B	3.19 B	0.59 A

Means followed by identical lowercase and uppercase letters (rows) did not differ according to the Tukey test ($p \leq 0.05$). Lowercase letters compare treatments. Capital letters correspond to time. *For the conventional process, the sugar concentrations are monitored during the initial and final time of the process (0-480 hrs). *Fruits recently harvested presented the same values for each treatment, corresponding to 0 hrs. There is no comparison between the treatments because they are freshly harvested fruits. SIAF: Self-Induced Anaerobiosis Fermentation.
ND = Not detected.

Table 2. Acids (g/kg) concentration in coffee before and at the end of processing by SIAF and conventional process in natural and pulped coffee.

Treatments	Time	Citric		Malic		Succinic		Lactic	
		Natural	Pulped	Natural	Pulped	Natural	Pulped	Natural	Pulped
Freshly harvested fruits	0 hrs	4.85 *A	3.01 *A	1.13 *A	1.74 *A	0.76 *A	1.17 *A	ND *C	ND *C
S. cerevisiae SIAF	96 hrs	1.93 aB	2.63 aA	0.25 bB	0.04 aB	0.41 cB	0.72 aB	0.65 bB	2.49 aB
C. parapsilosis SIAF		3.63 aA	2.36 aA	0.30 bB	0.06 aB	0.82 aA	0.84 aA	2.38 aB	1.82 bB
T. delbrueckii SIAF		2.20 aA	1.93 aA	0.25 bB	0.23 aB	0.53 cB	0.69 aA	0.65 bB	1.47 bB
Cont SIAF		4.85 aA	2.51 aA	0.72 aB	0.49 aB	0.64 bB	1.23 aA	1.07 bB	1.38 bB
S. cerevisiae SIAF	180 hrs	1.67 aB	2.57 aA	0.03 aC	ND aB	0.42 cB	0.35 aB	2.55 bA	5.91 bA
C. parapsilosis SIAF		2.73 aA	2.37 aA	0.05 aC	ND aB	0.74 bA	0.51 aA	3.57 aA	3.60 cA
T. delbrueckii SIAF		2.39 aA	2.46 aA	0.10 aB	0.07 aB	0.68 bA	0.82 aA	2.99 bA	2.79 dA
Cont SIAF		2.40 aA	3.05 aA	0.13 aC	0.15 aB	1.00 aA	0.89 aA	3.95 aA	8.33 aA
*Conventional Process	0 hrs	4.85 A	3.01 A	1.13 B	1.74 B	0.76 A	1.17 B	ND A	ND A
	480 hrs	3.13 A	4.04 A	2.02 A	2.75 A	1.26 A	2.22 A	ND A	ND A

Means followed by identical lowercase and uppercase letters (rows) did not differ according to the Tukey test ($p \leq 0.05$). Lowercase letters compare treatments. Capital letters correspond to time. *For the conventional process, the sugar concentrations are monitored during the initial and final time of the process (0-480 hrs). *Fruits recently harvested presented the same values for each treatment, corresponding to 0 hrs. There is no comparison between the treatments because they are freshly harvested fruits. SIAF: Self-Induced Anaerobiosis Fermentation.
ND = Not detected.

Table 3. Concentration of endo-β-mannanase enzymatic activity (pmol/min/g) in coffee before and at the end of processing by SIAF and conventional process in natural and pulped coffee.

Treatments	**Natural**	**Pulped**
0 hrs	3.57 aB	9.89 bA
96 hrs *S. cerevisiae* SIAF	0.60 bB	2.44 dA
96 hrs *C. parapsilosis* SIAF	0.68 bB	3.70 cA
96 hrs *T. delbrueckii* SIAF	0.76 bB	4.24 cA
96 hrs Control SIAF	1.07 bB	3.11 cA
180 hrs *S. cerevisiae* SIAF	0.50 bA	0.87 eA
180 hrs *C. parapsilosis* SIAF	0.63 bA	1.50 eA
180 hrs *T. delbrueckii* SIAF	0.52 bA	1.08 eA
180 hrs Control SIAF	1.06 bA	2.05 dA
480 hrs Conventional process	0.22 bB	10.99 aA

Means followed by identical uppercase and lowercase letters did not differ by the Scott–Knott test ($p \leq 0.05$). Lowercase letters compare treatments. Capital letters compare processing methods. *Freshly harvested fruits presented the same values for each treatment, corresponding to 0 hrs. There is no comparison between the treatments because they are freshly harvested fruits. SIAF: self-induced anaerobic fermentation.

Table 4. Seed viability (%) according to germination test (root protuberance at 30 days) at

SIAF sampling times 0 and 180 hrs for Natural and Pulped coffee.

Time	**Treatments**	**Natural**	**Pulped**
0 hrs	Freshly harvested fruits	94.00 *A	93.00 *A
180 hrs	*S. cerevisiae* SIAF	63.00 cC	42.00 bC
	C. parapsilosis SIAF	58.00 cC	58.00 aB
	T. delbrueckii SIAF	71.00 bB	59.00 aB
	Control SIAF	74.00 bB	62.00 aB
	Conventional process	85.00 aA	67.00 aB

Means followed by the identical lowercase and uppercase letters did not differ by the Tukey test ($p \leq 0.05$). Lowercase letters compare treatments. Capital letters correspond to time. * Freshly harvested fruits presented the same values for each treatment corresponding to time 0 hrs. *There is no comparison between the treatments because they are freshly harvested fruits. SIAF: self-induced anaerobiosis fermentation.