LUCIANA SILVA RIBEIRO

APPLICATION OF MICROORGANISMS FOR COFFEE FERMENTATION

LAVRAS – MG
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Tese apresentada à Universidade Federal de Lavras como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola do Departamento de Biologia para obtenção do título de Doutor.

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Co-orientadoras

LAVRAS – MG
2018

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RESUMO

Cafés arábica de três variedades apresentam resultados distintos quando inoculados com microrganismos. A qualidade do café pode ser melhorada através do uso de culturas iniciadoras na fermentação. A presença de microrganismos durante a fermentação do café interfere diretamente na qualidade e nas características da bebida final. O objetivo deste trabalho foi avaliar a o efeito na qualidade do café da inoculação de diferentes microrganismos e para, além disso, estudar a composição de cafés fermentados pela via úmida. Três variedades de café foram estudadas neste trabalho: Ouro amarelo, Mundo Novo e Catuaí vermelho, provenientes de duas regiões (Cerrado Mineiro e Sul de Minas Gerais). O primeiro aspecto abordado foi o efeito sensorial em duas variedades de café (Ouro Amarelo e Mundo Novo) processadas pelo método semi-seco e inoculadas com três cepas de leveduras (*Saccharomyces cerevisiae* CCMA 0200 e CCMA 0543; *Torulaspora delbrueckii* CCMA 0684). Foram usadas duas técnicas de análise sensorial (Prova de Xícara e Temporal dominance of sensations). Neste estudo foi possível observar que a variedade Ouro Amarelo obteve maiores notas para os atributos avaliados comparada com a variedade Mundo Novo. O uso das cepas CCMA 0543 e CCMA 0684 melhorou a qualidade sensorial das bebidas. O segundo aspecto abordado netes trabalho foi a diversidade e efeito da inoculação de bactérias na fermentação do café pela via úmida de processamento. Inicialmente foi estudada a diversidade bacteriana de três variedades (Ouro amarelo, Mundo Novo e Catuaí vermelho). Através de técnicas de identificação como MALDI-TOF (Matrix Assisted Laser Desorption/Ionization – Time of Flight) e sequenciamento foi possível identificar 41 espécies de bactérias. Além disso, foram quantificados alguns compostos alvo através de HPLC (Cromatografia líquida de alta performance) e CG-MS (Cromatografia gasosa acoplada ao espectrômetro de massas). *Lactobacillus plantarum* e *Leuconostoc mesenteroides* foram encontradas em todas as variedades. Ácido cítrico foi detectado em altas concentrações e as pricipais classes de voláteis encontradas foram ácidos, álcoois, aldeídos e hidrocarbonetos. O perfil sensorial demostrou a presença de sensações de acidez (Ouro amarelo e Catuaí vermelho), amargor, chocolate e nozes (Mundo novo) e doçura (Catuaí vermelho). E por fim, algumas espécies foram testadas quanto a sua capacidade fermentativa para serem aplicadas como cultura iniciadora em café da variedade Catuaí vermelho, fermentado em via úmida. Algumas cepas como *Pantoea dispersa* CCMA 1203; *Cellulosimicrobium cellulans* 1186, *L. mesenteroides* CCMA 1105 e *L. plantarum* CCMA 1065 apresentaram melhores resultados em relação a produção de ácidos e maior mudança no perfil volátil durante a fermentação. Estas cepas são fortes idicadoras para serem utilizadas como culturas iniciadoras em café fermentado pela via úmida. Portanto, conclui-se que a inoculação de microrganismos em café é uma alternativa altamente promissora, sendo dependente de variedade e tipo de processamento a ser aplicado.

Palavras-chave: Fermentação do café. Leveduras. Bactérias. Culturas iniciadoras. Cafés especiais. MALDI-TOF

ABSTRACT

Arabica coffees of three varieties present distinct results when inoculated with microorganisms. The attempt to improve coffee quality can be implemented at different stages of coffee production. Presence of microorganisms during coffee fermentation directly interferes with the quality and characteristics of final beverage. The aim of this study was to evaluate the effect on coffee quality of inoculation of different microorganisms and also to study a bacteriological composition of coffees processed by wet fermentation. Three varieties of coffees studied at work: Ouro amarelo, Mundo novo and Catuaí Vermelho, mining region (Cerrado Mineiro and Southern of Minas Gerais). Ouro amarelo and Novo mundo processed by semi-dry method inoculated with three yeast strains (*Saccharomyces cerevisiae* CCMA 0200 and CCMA 0543; *Torulaspora delbrueckii* CCMA 0684). Two sensory analysis techniques were used (Cup Test and Temporal Dominance of Sensations). It was possible to observe that Ouro Amarelo variety obtained higher scores for evaluated attributes. The use of the CCMA 0543 and CCMA 0684 strains improved the sensory quality of the beverages. The second aspect discussed in this work is the diversity and effect of the inoculation of bacteria in fermentation of coffee by the wet process. Initially, a bacterial diversity was studied (Ouro amarelo, Mundo Novo and Catuaí Vermelho). Through identification techniques such as MALDI-TOF (Array Assisted Laser Desorption / Ionization - Time of Flight) and sequencing it was possible to identify 41 species of bacteria. In addition, some target compounds were quantified through HPLC (High Performance Liquid Chromatography) and CG-MS (Gas Chromatography and a mass spectrometer). According to the results, *Lactobacillus plantarum* and *Leuconostoc mesenteroides* were found in all varieties. Citric acid was detected at high concentrations and as the main classes of volatiles found were acids, alcohols, aldehydes and hydrocarbons. The sensorial profile demonstrates the presence of sensations of acidity (Ouro amarelo and Catuaí vermelho), bitterness, chocolate and nuts (Novo mundo) and sweetness (Catuaí vermelho). And finally, some species tested for their fermentation capacity to be applied as a starter culture in coffee of the Catuaí vermelho, in wet fermentation process. Strains *Pantoea dispersa* CCMA 1203; *Cellulosimicrobium cellulans* 1186, *L. mesenteroides* CCMA 1105 and *L. plantarum* CCMA 1065 presented better results in relation to acid production and changes in volatile profile during fermentation. These strains are strongly selective to be as well as starter cultures in wet fermented coffee. However, it is concluded that the inoculation of microorganisms in coffee is a highly promising alternative, being dependent on the variety and type of processing to be applied.

Key words: Fermentation of coffee. Yeasts. Bacteria. Starter cultures. Specialty coffees. MALDI-TOF

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PRIMEIRA PARTE

1 INTRODUÇÃO

O café é uma das bebidas mais consumidas no mundo por milhões de pessoas rotineiramente. A produção de grãos de café causa importante impacto na economia do país, pois o Brasil é o maior produtor e exportador de café. A qualidade do café tem sido valorizada nos últimos anos dando credibilidade aos cafés especiais. Cafés especiais são produzidos quando todos os envolvidos na cadeia produtiva do café trabalham com padrões de excelência do início ao fim. São cafés que devem atender aos segmentos do mercado e aceitos por terem aroma, corpo, doçura, acidez e sabor equilibrados.

A fermentação pelo processamento via úmida tem como consequência a remoção da mucilagem ao redor do grão, facilitando assim a secagem. A mucilagem é composta por substâncias pécticas e outros açúcares. A degradação da mucilagem é realizada por enzimas endógenas dos frutos do café e pelos microrganismos presentes no processo.

A diversidade microbiana encontrada em café fermentado pela via úmida pode ser caracterizada pela predominância de bactérias e leveduras. A fermentação pela via úmida é rápida (48h) com rápido declínio do pH de 6 para 4.3.

O conhecimento da microbiota é de fundamental importância, assim como a função de cada um dos microrganismos na etapa de fermentação do café, para obtenção de culturas iniciadoras que proporcionem melhorias na qualidade final da bebida.

A inoculação de culturas iniciadoras objetiva padronizar o processo de fermentação e obter produtos com maior qualidade e segurança. A utilização de bactérias e leveduras na fermentação do café é uma alternativa economicamente viável obtendo um café diferenciado, agregando valor ao produto. Além disso, a utilização de culturas iniciadoras impacta de forma direta a produção de compostos responsáveis pelo aroma e sabor da bebida do café.

2 REFERENCIAL TEÓRICO

2.1 O café

O gênero *Coffea* pertence à família Rubiaceae, também conhecida como "a família do café", pois este é o representante mais conhecido. As duas principais espécies de cafeeiro cultivados no mundo para produção de bebida são *Coffea arabica* e *C. canephora* var. *robusta*. A planta selvagem (*C. arabica*) é originária da província de Kaffa na Etiópia (Península Arábica) em áreas de planalto com altitudes entre 1300 e 2000 metros da qual se disseminou para os países vizinhos e para o mundo todo durante o século XIII. Por outro lado, a *C. canephora* encontra-se amplamente distribuída na África tropical em altitudes abaixo de 1000 metros (MONACO et al., 1977; MURTHY; NAIDU, 2012; WINTGENS, 2009).

O fruto do cafeeiro consiste de uma casca exterior resistente e lisa chamada epicarpo, geralmente verde em frutos imaturos, mas que se torna vermelho-violeta ou vermelho escuro quando maduro. Em determinadas variedadespode se tornar amarelo ou laranja. O epicarpo cobre o mesocarpo amarelado, fibroso e doce, seguido por uma camada translúcida, incolor, fina, altamente viscosa e hidratada chamada de mucilagem (Figura 1). Internamente a esta camada existe um endocarpo fino de cor amarelada, conhecido como pergaminho. Por último, a película prateada cobre cada hemisfério do grão de café (endosperma) (De CASTRO; MARRACCINI, 2006; BELITZ; GROSCH; SCHIEBERLE, 2009; BERBERT et al., 2001; ESQUIVEL; JIMÉNEZ, 2012). As variedades de café podem apresentar diferenças no tamanho e forma dos grãos, porém, em geral os grãos apresentam aproximadamente 10 mm de comprimento e 6 mm de largura (WINTGENS, 2009).

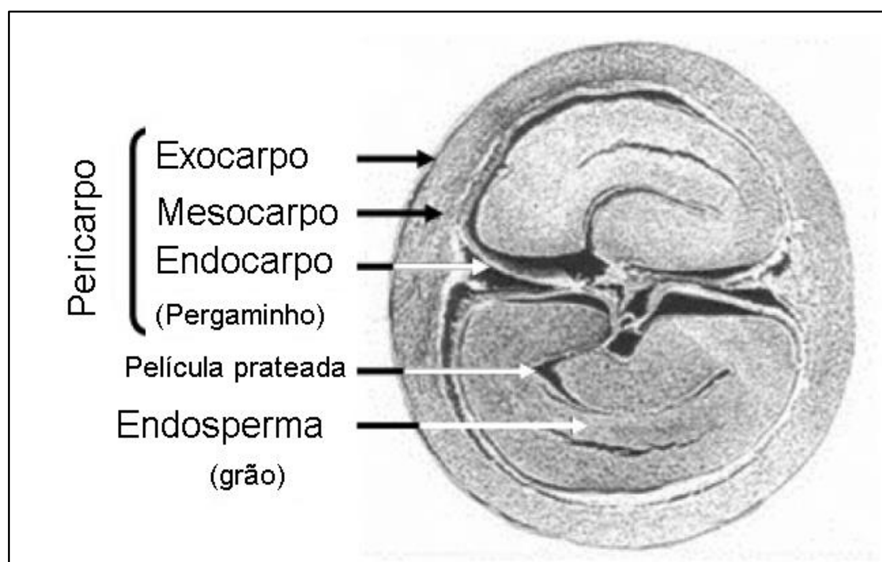
Os primeiros grãos de café introduzidos no Brasil vieram da Guiana em 1727 pertencentes a variedade Típica (*C. arabica* var. Typica Cramer). Após 125 anos, a variedade Bourbon Vermelho foi trazida ao país através da Ilha de Reunião, França. O Brasil é o principal produtor de café do mundo e cultiva plantas derivadas essencialmente destas duas variedades (CARVALHO, 2008; SAKIYAMA; FERRÃO, 2015).

Através de cruzamentos entre as cultivares surgiram nos anos seguintes variedades como Maragogipe, Amarelo de Botucatu e Bourbon Amarelo. Atualmente as cultivares mais importantes do Brasil são Mundo Novo (plantas altas), Catuaí

Vermelhoe Catuaí Amarelo (plantas baixas). A hibridização entre Sumatra e Bourbon Vermelho em 1943, originou a cultivar Mundo Novo e entre Mundo Novo e Caturra Amarelo foi originado o Catuaí Amarelo e Catuaí Vermelho em 1949 (SAKIYAMA; FERRÃO, 2015). As novas cultivares surgiram da necessidade de produzir plantas que aumentassem a produtividade, tamanho de grãos, vigor vegetativo, resistência a doenças e pragas e principalmente qualidade da bebida (CARVALHO, 2008).

Catuaí Amarelo e Mundo Novo são as variedades mais tradicionalmente cultivadas e foram hibridizadas com intuito de melhorar o vigor vegetativo das plantas originando plantas baixas como Topázio, Ouro Verde, Ouro Amarelo, Ouro Bronze e Travessia (BATISTA et al., 2016; SAKIYAMA; FERRÃO, 2015).

Figura 1. Representação esquemática dos tecidos presentes em frutos de *Coffea* sp no estádio maduro.



Fonte: Adaptado de De Castro e Marraccini (2006).

2.2 Economia e produção do café

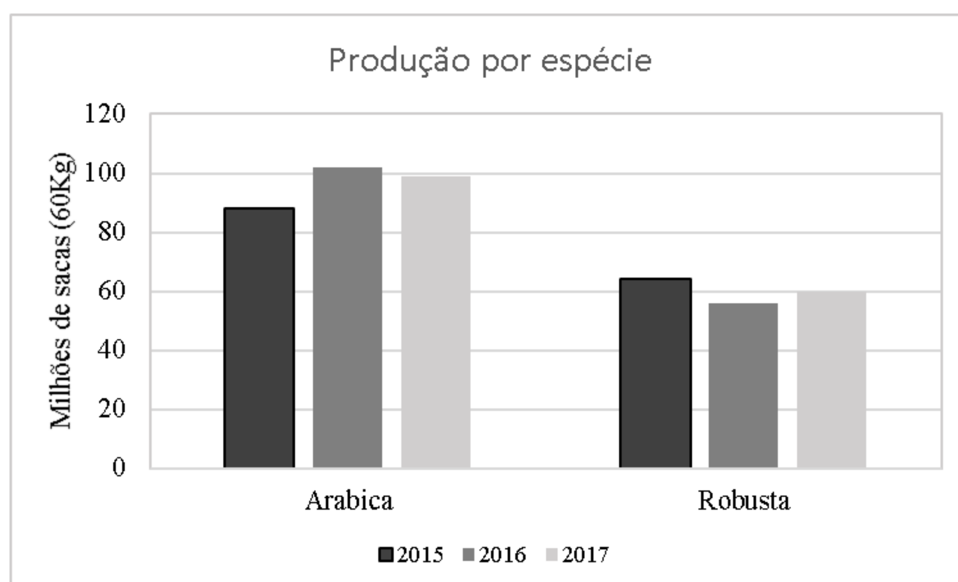
Devido à grande importância econômica do café, a Organização Internacional do café (ICO) foi criada em Londres em 1963 com objetivo de auxiliar os países importadores e exportadores do café com cotas de preços de mercado, preços ao produtor, produção, estoques e inventários (ICO, 2018). Depois do petróleo, o café é a mercadoria comercial mais valiosa do mundo, com vendas globais estimadas em US $ 90 bilhões. O café é o principal produto de exportação de alguns países como o Brasil, Uganda, Burundi, Ruanda e Etiópia. Cerca de 70% da safra mundial é cultivada em

pequenas propriedades menores que 10 ha e, portanto, é muitas vezes uma empresa familiar que fornece manutenção para mais de 25 milhões de pessoas em todo o mundo (BATISTA et al., 2016).

A importância econômica do café como bebida tem crescido a cada ano devido ao aumento do número de consumidores interessados em cafés especiais. A maior parte da bebida de café preparada no mundo é produzida a partir da espécie de café *Coffea arabica,* pois é considerada superior devido as apreciadas propriedades sensoriais (BERTRAND et al., 2003) e, portanto, alcança preços altos nos mercados de exportação brasileiro (GIELISSEN; GRAAFLAND, 2009). A produção mundial de café em 2017 foi de 159 milhões de sacas de 60 Kg, isso representou aumento de 0.8% em relação ao ano de 2016 (Figura 2). O Brasil é o principal produtor de café produzindo52 milhões de sacas no ano de 2017 (Figura 3) (ICO, 2018).

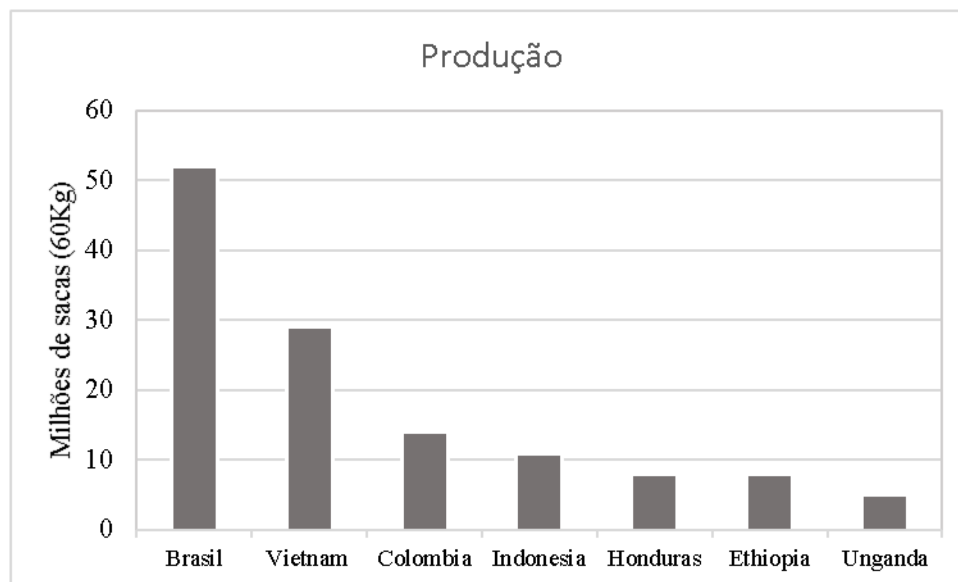
O desenvolvimento da produção de café, apesar de crescente no mundo, passou por períodos de oscilações. No início do século XX, o Brasil produziu 70% da produção mundial de café, no entanto com o crescimento da produção também em outras partes do mundo, isso representou em torno de 34% nos recentes anos (SAKIYAMA; FERRÃO, 2015). Rufino (2006) relatou que houve uma participação relativa dos países Latino-americanos nos anos 50, além do Brasil.

Figura 2. Produção mundial de café Arabica e Robusta.



Fonte: International Coffee Organization (2018).

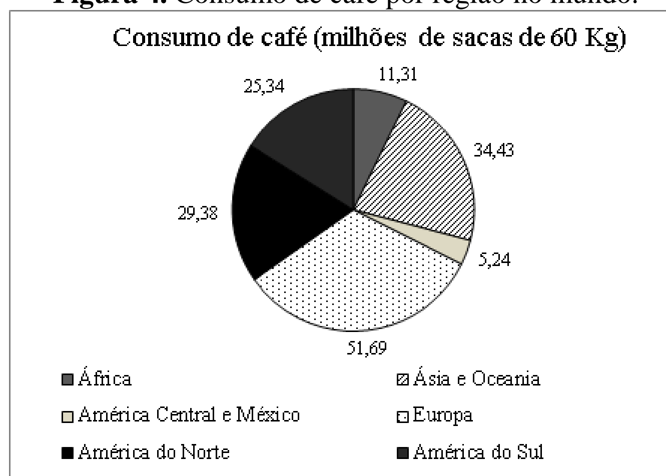
Figura 3. Produção total pelos principais países exportadores em 2018.



Fonte: International Coffee Organization (2018).

Estudos feitos pela Associação Brasileira de Indústrias de Café (ABIC, 2016) mostraram que a forma tradicional de fazer café com garrafa e filtro ainda é a mais popular no Brasil. Aproximadamente 81% dos consumidores preparam o café filtrado, enquanto 0,6% declaram utilizar preparação em monodoses ou cápsulas. Variações como os cafés gourmet têm sido mais procuradas pelos consumidores, existindo uma consistente evolução para atender aqueles mais atentos à diferenciação de regiões, sabores, certificações, entre outros (ABIC, 2016). O crescimento da produção, comércio e consumo do café demonstra claramente a importância do café na economia mundial (SAKIYAMA; FERRÃO, 2015). Estima-se que em 2016/2017 foram consumidas 157,3 milhões de sacas de 60Kg de café, sendo a Europa o maior consumidor (Figura 4) (ICO, 2018).

Figura 4. Consumo de café por região no mundo.



Fonte: ICO, 2016.

2.3 Composição química e formação de aromas no café

A bebida do café é o produto final mais importante obtido a partir do grão torrado e moído. Devido à importância da bebida de café em todo o mundo, extensas pesquisas são conduzidas para maior conhecimento da composição química, bem como do potencial benéfico e das propriedades prejudiciais do café (DÓREA; COSTA, 2005; HIDGON; FREI, 2006; MELETIS, 2006; NKONDJOCK, 2009; SERAFINI; TESTA, 2009; DÓREA; SISODIA, 2010).

A geração de sabor e aroma do café começa na planta, onde precursores de aromas são formados no café cereja e vão se tornando complexos ao longo das etapas de processamento e técnicas de preparação da bebida. Porém, a localização geográfica, genética da planta, método de colheita, clima, tipo de solo e sazonalidade estão entre os fatores que contribuem para a complexidade de aromas no café (Figura 5) (SUNARHARUM; WILLIAMS; SMYTH, 2014).

O café é muito conhecido pelas suas propriedades estimulantes atribuídas a cafeína, um típico alcalóide formado em frutos de cafés imaturos e gradualmente acumulada durante o desenvolvimento da semente (CHENG et al., 2016). No entanto, o número de compostos químicos identificados no grão é muito grande. Alguns compostos não voláteis estão presentes no café torrado e contribuem para a formação dos flavors. Dentre estes estão os alcalóides, ácidos clorogênicos, ácidos carboxílicos, carboidratos, lipídeos e proteínas (BUFFO; CARDELLI-FREIRE, 2004).

Acredita-se que a cafeína, um metabólito secundário nitrogenado, influencie na percepção do corpo e amargura de um café fresco. Os conteúdos de cafeína podem

variar com as cultivares. O café Arabica é muito popular devido ao seu baixo conteúdo de cafeína comparado com o café Robusta, concentrando 0,6 a 1,8% e 1,2a4,0%, respectivamente (BICHO et al., 2013; HECIMOVIC et al., 2011; VIANI, 1993; SUNARHARUM et al., 2014).

O segundo alcalóide mais encontrado em cafés é a trigonelina e seus dois derivados, o ácido nicotínico e N-metilnicotinamida. A trigonelina está presente no café em concentrações que variam de 0,3 a 1,3%contribuindo para a percepção aromática global da bebida de café e pelo amargor. Aproximadamente de 60 a 90% deste composto é degradado durante a torrefação do café. Apesar disso, contribui para desejáveis aromas que são formados como pirazinas, furanos, alquil-piridinas e pirróis (CHENG et al., 2016; LEE et al., 2015; OESTREICH-JANZEN, 2010).

Os ácidos clorogênicos (CGAs) são um grupo de compostos fenólicos da família dos estéres formados pela degradação de ácidos trans-cinâmicosformando o ácido cafeíco, ferúlico, lactonas e outros derivados de fenóis. Estes compostos podem contribuir paraa percepção da adstringência e amargor da bebida (UPADHYAY; MOHAN RAO, 2013; LEE et al., 2015; OESTREICH-JANZEN, 2010). Os principais subgrupos de CGAs encontrados em grãos de café verde são ácidos cafeoil-quínico (CQA), ácidos dicafeoilquínico (diCQA) e ácidos feruloilquínico (FQA) (CLIFFORD, 1999).

Os ácidos alifáticos não voláteis como ácido cítrico, málico, clorogênico e quínico compreendem em torno de 11 a 6% do grão verde e torrado, respectivamente. Durante a torrefação estes ácidos formam outros compostos como lactonas e fenóis voláteis como guaiacol e 4-vinilguaiacol. Ácidos voláteis também estão presentes, como acético, propanóico, butanóico, isovalérico, hexanóico e decanóico no grão verde e são precursores de aromas que serão gerados após a torrefação (ARYA; RAO, 2007; BELITZ; GROSCH; SCHIEBERLE, 2009; GONZÁLEZ-RÍOS et al., 2007a; SUNARHARUM et al., 2014).

Os polissacarídeos são encontrados em diversas formas no café, como na maioria dos tecidos vegetais. Nos grãos de café verde são encontrados polissacarídeos insolúveis, na forma de arabinogalactanas, mananas, celulose e hemicelulose (BRADBURY, 2001). Polissacarídeos e açúcares simples, também encontrados no grão, são responsáveis pela formação de produtos de caramelização e reações de Maillard. Estes desempenham importante papel na formação de aromas e também contribuem para a percepção da viscosidade da bebida (BUFFO; CARDELLI-FREIRE,

2004; LEE et al., 2015). No momento da torrefação, os açúcares redutores gerados pela hidrólise de sacarose juntamente com os aminoácidos, são responsáveis pela reação de Maillard. A partir dessa reação são formados compostos voláteis importantes na formação do flavor do café como furanos, pirazinas, ácidos alifáticos, tióis, furanonas, tiofenos e hidroximetilfurfural que compõe descrições sensoriais de caramelo, torrado, "coffee-like", amêndoas, nozes, cacau e pão torrado (GROSCH, 2001; FARAH et al., 2006; LEE et al., 2015; LEE et al. 2016a).

Os lipídeos encontrados em café verde variam entre 7 e 17%. A hidrolise de lipídeos em ácidos graxos leva a formação de metabólitos secundários como ésteres, metil cetonas, álcoois e lactonas. A viscosidade da bebida é um parâmetro atribuído a presença de lipídeos (OESTREICH-JANZEN, 2010; LEE et al., 2015).

O conteúdo de proteínas e aminoácidos livres desempenha importante papel na formação do flavor, pois durante a torrefação ocorre a reação de Maillard ou caramelização. O grupamento amina dos aminoácidos livres ou compostos de conteúdo nitrogenado reage com o grupo carboxil dos açúcares redutores, hidroxi-ácidos e fenóis para produzir aminoaldoses e aminocetonaspor condensação. Como resultado, o café apresenta melanoidinas que dão cor acastanhada e outros componentes são formados, tais como compostos heterocíclicos contendo nitrogênio e enxofre (BUFFO; CARDELLI-FREIE, 2004; ALVES et al., 2010; LEE et al., 2015).

Compostos voláteis produzidos durante a torrefação são indiscutívelmente os maiores colaboradores para percepção da qualidade aromática do café. As classes químicas de compostos encontrados no café incluem hidrocarbonetos, álcoois, aldeídos, cetonas, ácidos carboxílicos, esters, pirzinas, pirroles, piridinas, compostos sulfurados, furanos, fenóis, entre outros. Quantitativamente os mais significantes são furanos e pirazinas, enquanto qualitativamente os compostos sulfurados juntamente com as pirazinas são mais significantes para o flavor do café (LEE et al., 2015).

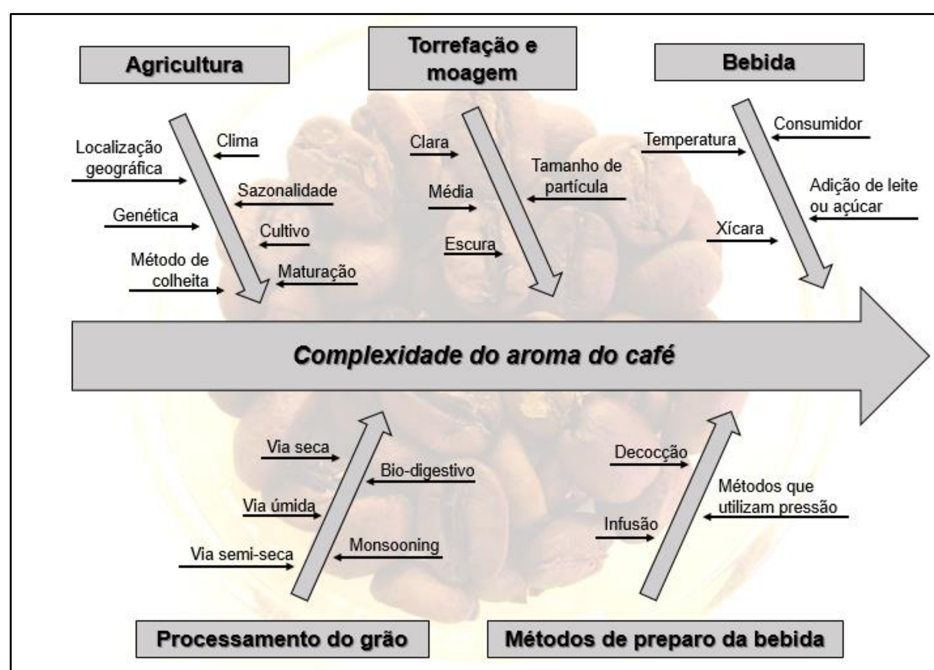
Todos estes compostos citados contribuem para formação do flavor do café. O flavor pode ser descrito como uma combinação entre aroma, sabor, textura e sensação na boca de um alimento. O aroma é um atributo mais importante no flavor do café. O flavor é um componente olfatório do sabor percebido retronasalmente. Esta percepção retonasal ocorre com alimentos voláteis que fluem da boca através da parte posterior da garganta alcançam a cavidade nasal através da faringe. Neste local os voláteis interagem com os receptores no epitélio olfativo, gerando estímulos nervosos transmitidos para o cérebro, que então processa a informação sensorial como reconhecimento de odor. No

entanto, a percepção de sabor humana é detectada nos receptores encontrados na língua e podem ser definidos como cinco gostos básicos, doce, amargo, salgado, azedo e umami (SUNARHARUM et al., 2014; NOBLE, 1996).

Vários fatores influenciam a geração do flavor do café (Figura 5), tais como fatores ambientais, origem geográfica, clima, altitude e temperatura, fatores nutricionais e de desenvolvimento da semente, boas práticas de agricultura, técnicas de processamento e estocagem e o preparo da bebida (IAMANAKA et al., 2014; SUNARHARUM; WILLIAMS; SMYTH, 2014). A torrefação tem influência significante no flavor do café e tem sido alvo de muitas pesquisas (BUFFO; CARDELLI-FREIRE, 2004; ESQUIVEL; JIMÉNEZ, 2012).

A interação positiva entre genótipo e ambiente pode resultar em um café de excelente qualidade e flavor. Para aprimorar a qualidade do café, é essencial o entendimento do metabolismo e dos genes que governam o acúmulo de compostos precursores de flavors durante o desenvolvimento do grão. Numerosos estudos têm sido conduzidos com esse propósito, especialmente com relação às modificações que ocorrem com os constituintes bioquímicos não voláteis (CHENG et al., 2016; LEE et al., 2015). A qualidade de aromas superiores pode ser direcionada através da utilização de culturas iniciadoras (EVANGELISTA et al., 2014a, b; PEREIRA et al., 2014).

Figura 5. Fatores que influenciam a qualidade do aroma e sabor do café.



Fonte: adaptado de SUNARHARUM; WILLIAMS; SMYTH (2014).

2.4 Etapas para produção de café

A qualidade do café utilizado para produzir bebidas está relacionada com a composição química dos cafés torrados e estes estão diretamente ligados às condições de colheita e pós-colheita como secagem, processamento e estocagem (FRANCA, 2005b).

A colheitaé influenciada pelo mercado consumidor alvo, pelo processamento ao qual será submetido e pela viabilidade e custo disponível para trabalho. O ideal é que a colheita seja realizada com a maior parte dos frutos no estágio adequado de maturação (cafés cereja), com a menor possibilidade de dano à planta, independente do processamento a ser utilizado. A colheita pode ser realizada por derriça total, onde os frutos são selecionados, recolhendo os grãos manualmente, sem seleção de apenas frutos maduros. O resultado são lotes com diferentes estádios de maturação. Na colheita mecânica se utilizam máquinas acopladas a tratores, compostas de pás que tremem ao longo das fileiras fazendo com que caiam os frutos maduros, porém não impede que caiam também frutos em estádios avançados de maturação ou imaturos (BORÉM, 2008; BRANDO, 2009; BATISTA et al., 2016).

Após o processamento o café deve ser levado para os pátios de secagem até atingirem 11-12% de umidade. A secagem é um processo complexo e crucial na qualidade do café, pois como qualquer vegetal após a colheita mantém as propriedades de uma planta viva, incluindo a respiração e transpiração por um tempo (ROJAS, 2009). A taxa de secagem do pergaminho é dependente da umidade inicial, temperatura e umidade do ambiente, espessura do espalhamento do café nos pátios de secagem e periodicidade de agitação (MURTHY; NAIDU, 2012).

Os grãos passam por um processo de torrefação em altas temperaturas (200-300 ºC) com constante agitação para assegurar que todos os grãos sejam torrados igualmente. Este processo é crítico na produção da bebida, pois os aromas e sabores são dependes desta etapa. Durante a torrefação, a cor verde dos grãos passa a amarelo, e então a marrom-claro e mais tarde para marrom-escuro. Os açúcares do grão são transformados em CO_2, reações de Maillard, degradação de Strecker e reações de pirólise são responsáveis pela formação do complexo aroma do café. (BAGDONAITE; DERLER; MURKOVIC, 2008; NARITA; INOUYE, 2014).

2.5 Técnicas de processamento do café

Os frutos do café podem ser processados por três métodos distintos: via natural ou seca, via semi-seca e via úmida. Os diferentes tipos de processamento podem produzir bebidas com sabores e aromas particulares das quais o consumidor pode escolher de acordo com suas preferências (VILELA et al., 2010). Assim, o tipo de processamento realizado depende de fatores como disponibilidade de infraestrutura, recursos financeiros, relação custo benefício do método, atendimento à legislação ambiental e padrão desejado de qualidade (BORÉM, 2008).

Em todos os processamentos do café ocorre uma fermentação espontânea pelos microrganismos provenientes dos frutos e do ambiente. A fermentação do café é uma etapa no qual a mucilagem é degradada enquanto os grãos são simultaneamente secos até atingir 11-12% de umidade (SCHWAN; WHEALS, 2003). O tempo requerido para a fermentação difere entre os métodos de processamentos. Durante a fermentação uma série de reações fisiológicas ocorre nos grãos, como a redução da umidade, açúcares simples são transformados e há a formação de aroma e sabor (SILVA et al., 2013; VAAST et al., 2006). A secagem do café é uma etapa importante, uma vez que se não estiver seco corretamente, torna-se frágil e produz muitos grãos quebrados na etapa de beneficiamento. Este tipo de café fica propenso a contaminação e deterioração por fungos e bactérias indesejáveis na estocagem (BATISTA et al., 2016).

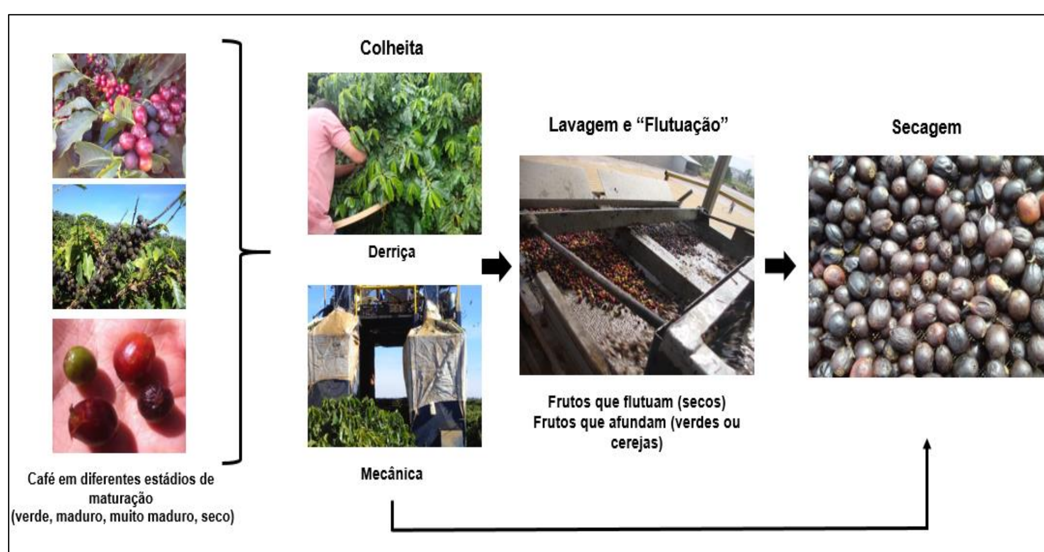
O processamento do café requer um elevado grau de conhecimento, pois grande quantidade de subprodutos é formada, tais como a polpa, casca do café, pergaminho, película prateada e águas residuárias, que devem ser dispostos no meio ambiente corretamente ou utilizados como matérias primas para outros produtos como fertilizantes, alimentação de gado, compostagem, entre outros (MURTHY; NAIDU, 2012).

2.5.1 Processamento via seca ou natural

O processamento via seca, resulta no chamado café natural ou não lavado. O fluxograma desta forma de processamento está demonstrado na figura 6. Este é o método mais antigo e simples de processamento do café. Este processamento implica que o fruto do café seja seco íntegro (polpa, pergaminho e grão) (BRANDO; BRANDO, 2015). Muitos países com chuvas escassas e longos períodos de estiagem e sol utilizam

este método. Cerca de 90% do café arábica produzido no Brasil é processado por este método. A via seca envolve a fermentação do fruto inteiro e, geralmente, produz um café que tem corpo acentuado, é doce, suave e complexo. Os frutos de café (verde, cereja e seco) são espalhados em plataformas deconcreto ou asfaltoem camadas de aproximadamente 5- 10 centímetros de espessura, amontoados à noite e espalhados durante o dia. Ao longo de 10-25 dias de secagem ao sol, ocorre a fermentação microbiana de forma natural (SCHWAN; WHEALS, 2003; SILVA et al., 2000; SILVA et al., 2008).

Figura 6. Processamento seco ou natural do café.



Fonte: Do autor (2018).

Os frutos do café devem ser separados de acordo com os diferentes estádios de maturação com intuito de obter a maior qualidade possível, independentemente do processo ao qual serão submetidos. Porém, muitos produtores ignoram esta etapa e levam o café diretamente da colheita para a secagem sem nenhum tipo de separação prévia. O resultado é um produto de baixa qualidade. O café pode ser limpo por separação hidráulica, de acordo com densidade do grão que é em função do teor de umidade. Os grãos secos, brocados, folhas e paus secos flutuam na água e os grãos verdes, maduros e pedras afundam (BRANDO; BRANDO, 2015).

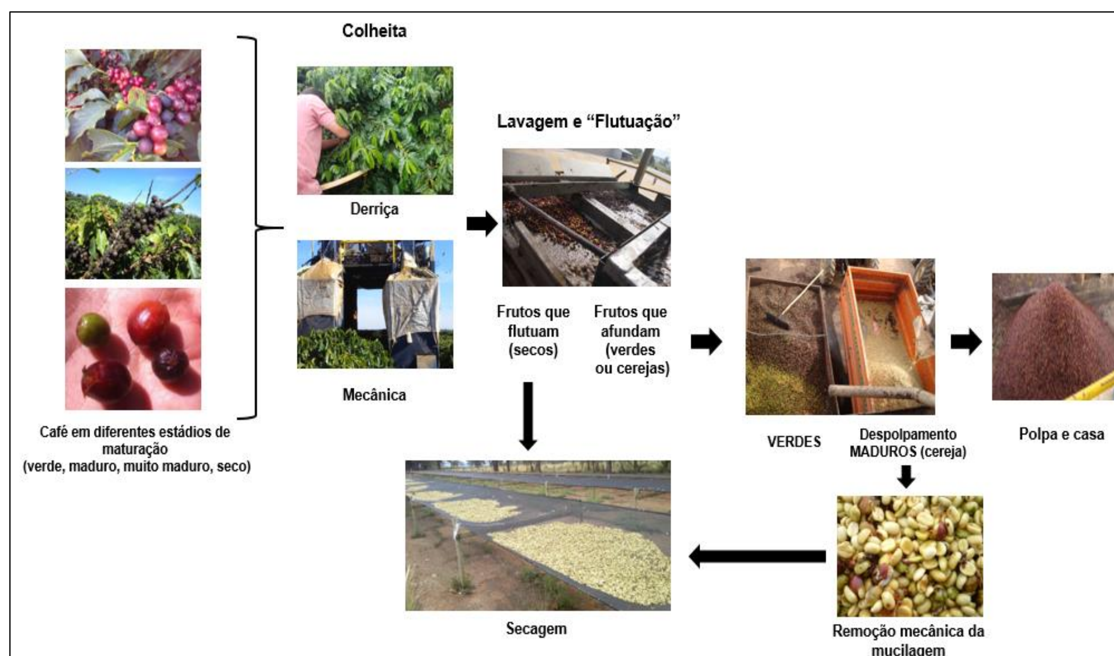
Este tipo de processamento produz um café que tem mercado próprio, pois sua bebida é encorpada e menos ácida do que a produzida pelo método da via úmida (BATISTA et al., 2016).

2.5.2 Processamento via semi-seca

O processamento semi-seco se originou no Brasil nos anos 90 e é também chamado de “cereja descascado” ou simplesmente CD esuas etapas de processamento sãomostradas na figura 7. A utilização do café cereja despolpado foi indicada como alternativa para resolver o problema da mistura de frutos maduros e imaturos encontrados no processamento natural e acelerar e facilitar a secagem. O processamento semi-seco visa a separação de cafés cereja maduros dos imaturos quando não é feita a colheita seletiva (BRANDO; BRANDO, 2015). Os grãos verdes produzidos desta forma são usualmente usados em blends de espresso. Alguns autores afirmam que este processo é uma variação e combinação do processamento úmido visto que os frutos do café também são descascados, porém na via úmida estes são fermentados em plataformas imersos em água e não são espalhados diretamente em pátios de cimento (SILVA et al., 2013; VILELA et al., 2010; BATISTA et al., 2016).

Neste processo originam-se três tipos de café: quando se remove a casca mecanicamente e a mucilagem é removida por fermentação biológica, tem-se o café despolpado; quando se remove mecanicamente a casca e parte da mucilagem, tem-se o café cereja descascado (CD); e quando se remove a casca e a mucilagem, tem-se o café desmucilado (BORÉM, 2008).

Figura 7. Processamento semi-seco do café.



Fonte: Do autor (2018).

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As despolpadeiras (tipo tambor, disco, tambor espiral vertical) e o fornecimento adequado de água limpa são necessários para despolpamento. A remoção total ou parcial da mucilagem tem como objetivo facilitar o revolvimento do café nos pátios e reduzir o tempo de secagem produzindo cafés de alta qualidade com características organolépticas distintas (AVALLONE et al., 2000; BRANDO; BRANDO, 2015; MURTHY; BASAVARAJ; NAIDU, 2001).

2.5.3 Processamento via úmida

No processamento via úmida, após o café ser lavado, o fruto cereja tem a casca e a polpa removidas mecanicamente. O mesocarpo remanescente é chamado de mucilagem, fica aderida ao pergaminho e é removido durante a fermentação em tanques com água (BRANDO; BRANDO, 2015; BRANDO 2009) (Figura 8). Na Colômbia, América Central e Hawai, o processamento úmido é usado para café Arábica (SCHWAN; WHEALS, 2003). O método de processamento via úmidamanteve-se praticamente o mesmo até o início dos anos 1980. Apenas nos últimos 30 anos este processamento tem sofrido mudanças, pois esse método gera uma grande quantidade de produtos residuais principalmente água. A reconversão de algumas tecnologias tem sido feita para uma nova abordagem ecológica que requer menos uso de água e menor dano ambiental nos últimos 20 anos (BRANDO; BRANDO, 2015; BRANDO, 2009).

Figura 8. Processamento úmido do café.

Fonte: Do autor (2018).

O principal objetivo deste método é a remoção da mucilagem dos grãos de café através da fermentação realizada pelas enzimas microbianas. A quantidade de água utilizada nesta técnica pode ser diminuída com a utilização de despolpamento a seco e reuso da água para outros fins (AVALLONE et al., 2001a; BRANDO; BRANDO, 2015).

A fermentação ocorre de forma natural, em tanques de concreto nos quais devem ser considerados o tamanho e forma. O sistema mais usado é a de forma retangular com um fundo inclinado, usado tanto como um tanque e meio de transporte para a saída do café. O tamanho é dependente da capacidade de produção da fazenda, pois o tanque deve ser preenchido rapidamente (1 hora) para evitar fermentações indesejáveis e heterogeneidade entre o primeiro e último grão a ser fermentado. O café deve ser recoberto com água limpa e durante a fermentação, a mucilagem é hidrolisada por enzimas endógenas produzidas por microrganismos naturalmente presentes nos frutos do café (BRANDO, 2009; AGATE; BHAT, 1966; MASOUD; JESPERSEN, 2006; VAAST et al., 2006).

O tempo de fermentação pode variar substancialmente, de 6 até 72 horas, dependendo de alguns fatores e principalmente da temperatura ambiente. Temperaturas mais altas (25-30ºC) aceleram o processo e a remoção de mucilagem é mais rápida. A fermentação é finalizada quando toda a mucilagem é removida. Não existe um método cientifico para verificação, esta é feita de modo empírico pela fricção de alguns grãos na mão. Um alto rangido deve ser ouvido claramente como fricção de pedras então a fermentação está finalizada. Os grãos perdem a textura mais viscosa e adquire a sensação mais áspera nas mãos. Os cafés fermentados devem ser lavados imediatamente para evitar fermentação excessiva que causa a formação de sabores estranhos e afeta a qualidade (BRANDO; BRANDO, 2015; BRANDO, 2009; BATISTA et al., 2016).

Os grãos resultantes ainda cobertos pelo pergaminho são secos geralmente em terreiro suspensos e após a secagem são beneficiados e a película prateada também pode ser removidapor polimento, produzindo cafés de alta qualidade (BELITZ et al., 2009; GONZÁLEZ-RÍOS et al., 2007b; JOËT et al., 2010a).

2.6 Microbiota presente no café

O propósito da fermentação natural do café é a quebra da camada de mucilagem, que é rica em polissacarídeos (pectinas) e substâncias menos complexaspara formação dos precursores do sabor e aroma (BATISTA et al., 2016). Frutos de café contêm enzimas endógenas que degradam a camada de mucilagem, mas essa atividade não é suficiente para um processo completo e adequado da remoção da mucilagem (AGATE; BHAT, 1966). A mucilagem é degrada pela fermentação que permite o crescimento de microrganismos produtores de enzimas tais como poligalacturonase (PG) que catalisa a hidrólise de ligações 1,4-glicosídicas em ácido poligalacturônico; pectina liase (PL) que atua catalisando dissociação da pectina liberando ácidos galacturônicosinsaturados. A terceira enzima é pectina metilesterase (PME) responsável pela desesterificação do grupo metoxila da pectina formando ácido péctico e metanol (AGATE; BHAT, 1966; MASOUD; JESPERSEN, 2006; SILVA et al., 2013).

Os microrganismos estão presentes naturalmente em todas as etapas de pré e pós-colheita e podem influenciar a qualidade da bebida. Os grãos de café possuem naturalmente precursores necessários para gerar aromas e sabores típicos durante a torrefação, porém a microbiota natural presente durante a fermentação/secagem confere aromas especiais na bebida do café. O conhecimento das espécies microbianas dominantes no café e no processamento éextremamente relevante para obtenção de bebidas de qualidade (VILELA et al., 2010).

Os microrganismos também estão associados com a formação de álcoois e ácidos, particularmente ácidos acético, láctico, butírico e outros ácidos carboxílicos de cadeia longa e podem conferir flavor característico ao café (SILVA et al., 2013).

A microbiota presente em frutos do cafeeiro incluem leveduras, fungos filamentosos e bactérias (SILVAet al., 2000, 2008; SAKIYAMA et al., 2001; MASOUD et al., 2004; MASOUD; JESPERSEN, 2006; De BRUYNE et al., 2007; VILELA et al., 2010).

A biodiversidade microbiana do café varia em número e espécie, depende da variedade de café, propriedades físicas e químicas do epicarpo, método de processamento, fatores ambientais da região em que são cultivadas, como umidade, temperatura e da população do solo.

Silva (2015) fez um relato cronológico dos estudos realizados sobre a degradação da mucilagem. Este relato mostra que questões sobre a ação microbiana no café se iniciaram em 1910 quando Gorter (citado por FRANCO, 1960) reportou que a degradação da mucilagem era realizada pela ação dos microrganismos, porém após

alguns anos, Lilienfeld-Toal (1931) e outros autores afirmaram que a degradação era realizada apenas por enzimas do próprio fruto do café. Franco (1960) elaborou um experimento com frutos do café despolpados e esterilizados, mantendo-os em meio estéril por 33 dias. O autor observou que não houve degradação da mucilagem, confirmando a necessidade da ação microbiana.

Pesquisadores tem se dedicado nos últimos anos a estudar o café com intuito de conhecer a microbiota presente e seu papel, porém ainda não é consenso entre os pesquisadores o impacto destes sobre o processamento e qualidade do café (SILVA et al., 2000; SILVA et al., 2008; BATISTA et al., 2009; EVANGELISTA et al., 2014a, b; SCHWAN; FLEET, 2015).

2.6.1 Bactérias

A diversidade de bactérias está intimamente relacionada com o tipo de processamento conduzido para fermentar o café (SILVA, 2015). Silva et al. (2000) estudaram a diversidade de microrganismos durante a maturação e processamento natural do café e observou que em todas as etapas o grupo das bactérias era o mais abundante em termos de espécies, seguido pelos fungos filamentosos e por último as leveduras. A microbiota inicial neste processo é composta extensivamente por bactérias Gram-positivas e Gram-negativas, com o passar do tempo o fruto vai perdendo umidade e a atividade de água fica mais baixa, sendo susceptível ao acometimento de leveduras e fungos (SILVA et al., 2008).

Bactérias endofíticas foram isoladas a partir de grãos de cafédesinfetados. *Paenibacillus amylolyticus*foi identificado como estirpe fortemente produtora de pectinases (SAKIYAMA et al., 2001). Avallone et al. (2001a, b) observaram que a maioria das bactérias encontradas em café no México eram bacilos Gram-negativos. As cepas isoladas com alta frequência em meio contendo pectina de maçã eram coliformes amplamente distribuídos no ambiente (73% *Klebsiella* e 28% *Erwinia*) mesmo que estes não tenham sido reportados como cepas produtoras de pectinases. Ao longo da fermentação de café, 60% dos açúcares simples foram degradados pelo total de microbiota e não especificamente por microrganismos com atividade pectinolítica.

No processamento semi-seco, Vilela et al. (2010) isolaram e identificaram espécies de bactérias como *Bacillus subtilis, Escherichia coli, Enterobacter agglomerans, Bacillus cereus* e *Klebsiella pneumoniae*.

Os gêneros *Leuconostoc, Weissella* e *Lactobacillus* foram identificados a partir da fermentação de café pela via úmida (EVANGELISTA et al., 2015). *Leuconostoc mesenteroides*, *Leuconostoc citreum*, *Leuconostoc pseudomesenteroides*, *Lactobacillus plantarum* e *Weissella soli*foram as bactérias ácido láticas mais abundantes encontrado em estudos da diversidade em café cereja e processado pela via semi-seca (EVANGELISTA et al., 2014a). Recentemente, foi observado, que as bactérias do ácido lático predominam o processamento úmido (SCHILLINGER et al., 2008; LEONG et al., 2014;; BATISTA et al., 2016).

2.6.2 Fungos filamentosos

Desde 1900, muitos autores reportam a ocorrência de fungos filamentosos em grãos de café. Inicialmente, a preocupação principal era a contribuição com sabores estranhos do café. Mas nos últimos anos esta preocupação tem sido focada na produção de micotoxinas, em especial a ocratoxina A (OTA) (SILVA, 2015).

A ocratoxina A (OTA) é a toxina mais prevalente em café. As micotoxinas são metabólitos secundários produzidas por alguns fungos filamentosos, entre estes se destacam os gêneros *Aspergillus* e *Penicillium* (VELMOUROUGANE et al., 2011; LEONG et al., 2014). A ocratoxina A (OTA) tem sido estudada por ser nefrotóxica, hepatotóxica, teratogênica, cancerígena e imunossupressora (PARDO et al., 2005; ROJAS, 2009).

Existem espécies que produzem a OTA que foram encontrados em associação com café: *Penicillium verrucosum, P. nordicum, Aspergillus niger, A. ochraceus*, *A. carbonariouse* *A. westerdijikiae* (JOOSTEN et al, 2001; VELMOUROUGANE et al., 2011; IAMANAKA et al., 2014). A produção de micotoxinas pode acontecer nos grãos de café antes da colheita, pois fungos filamentosos têm sido isolados a partir da superfície do fruto e durante o processamento do café (ROUSSOS et al, 1995; SILVA et al., 2000) e pode ocorrer ao longo de processamento, pós-colheita, armazenagem e transporte (SUÁREZ-QUIROZ et al., 2004).

Geralmente a ocorrência de fungos filamentosos em café está associada a aspectos negativos e redução da qualidade da bebida, exceto por *Cladosporium* que é frequentemente encontrado em grãos de boa qualidade (BITANCOURT, 1957a, b; ALVES; De CASTRO, 1998).

Não existem relatos sobre a ocorrência de fungos na via úmida. Isso é esperado, uma vez que processo dura apenas 48 horas, aproximadamente, em um ambiente com alta atividade de água, sendo, mais susceptível ao crescimento de bactérias e leveduras. As bactérias e leveduras crescem mais rápidas e competem por nutrientes e energia com os fungos filamentos. Assim como os fungos, a presença de ocratoxina A (OTA) neste ambiente é baixa, pois como os grãos de café estão imersos em água dentro dos tanques, favorece condições anaeróbicas de crescimento. A maior detecção de fungos nesse tipo de processamento ocorre na etapa de secagem, especialmente se houver mucilagem residual nos grãos a serem secos (SILVA, 2015).

2.6.3 Leveduras

As leveduras sãodescritas com grande importância em muitos estudos da via seca e semi-seca, pois muitas cepas são produtoras de enzimas pectinolíticas. Isso ocorre devido as condições favoráveis de crescimento nestes ambientes (SILVA et al., 2000; VAN PEE; CASTELEIN, 1971; AVALLONE et al., 2001a; AGATE; BHAT, 1966).

Silva et al. (2000; 2008) isolaram e identificaram leveduras em fermentações pelo processamento seco. Os gêneros comumente encontrados foram *Pichia, Candida, Arxula, Saccharomycopsis*e *Debaryomyces*.

Durante o processamento úmido do café arábica na Tanzânia foram identificadas por Masoud et al. (2004) leveduras em diferentes estádios de maturação da seguinte forma: *Pichia anomala* predominou sobre os cafés cerejas, polpas, grãos frescos durante o primeiro dia de fermentação. Essa estirpe foi também isolada durante o processo de secagem. *Hanseniaspora uvarum* foi predominante sobre café cereja e durante a fermentação. *Pichia kluyveri* foi predominante durante a fermentação e secagem. Outras espécies isoladas foram: *Torulaspora delbrueckii, Kluyveromyces marxianus*, *Candida pseudonitermedia, Pichia ohmeri* e *Issatchenkia orientalis*.

Dentre as espécies isoladas da via úmida *Pichia kluyveri* e *P. anomala* mostraram alta atividade pectinolítica. *Hanseniaspora uvarum*apresentou atividade de poligalacturonase, indicando que estas espécies podemestar envolvidas na hidrólise da celulose e pectina da mucilagem no café (MASOUD; JESPERSEN, 2006).

As espécies de leveduras *Torulaspora delbrueckii*, *Rhodotorula mucilaginosa*, *Saccharomyces bayanus*, *H. uvarum* e *Kloeckera* sp. foram identificadas durante o

processo de fermentaçãopela via semi-seca. O gênero *Kloeckera* é um grupo de levedura que está geralmente presente na fermentação vegetal com baixo teor de álcool. Espécies de *Candida* estavam presentes em uma altapopulação após 96 h de fermentação, e essas leveduras podem tolerar condições de baixa atividade de água (0,6 e 0,7) (BARNETT et al., 2000), que é uma característica da fase de secagem do processamento.

2.7 Culturas iniciadoras em café

Os primeiros relatos de potenciais culturas iniciadoras para café foram feitos por Massawe e Lifa (2010). Neste estudo os autores testaram duas espécies de leveduras (*Pichia anomala* e *Pichia kluyveri*) e quatro gêneros de bactérias láticas (*Leuconostoc, Weissella, Lactobacillus* e *Enterococcus*) como culturas iniciadoras em café fermentado pela via úmida. Posteriormente, Silva e colaboradores (2013), selecionaram bactérias e leveduras pectinolíticas e testaram quanto à performance fermentativa em meio contendo parte do mesocapo do café. Foram utilizados isolados de *Bacillus cereus, Bacillus megaterium, Bacillus subtilis, Candida parapsilosis, Pichia caribbica, Pichia guilliermondii* e *Saccharomyces cerevisiae*. As cepas *S. cerevisiae* CCMA 0543 (antiga UFLACN727), *P. guilliermondii* UFLACN731 e *C. parapsilosis* CCMA 0544 (antiga UFLACN448) foram considerados candidatas promissoras a serem testadas como culturas iniciadoras em café. Posteriormente, Evangelista et al. (2014a, b) testaram estas leveduras em café fermentado pela via seca e pela via semi-seca. Assim, os autores afirmam que as leveduras inoculadas persistem durante toda a fermentação e resultam em uma bebida com um sabor característico (caramelo e frutado) e boa qualidade sensorial.

No processo fermentativo pela via úmida do café também foram testadas culturas iniciadoras. Pereira et al. (2014, 2015a, b), avaliaram o potencial de nove cepas de leveduras na produção de compostos aromáticos em meio de polpa de café e a respectiva atividade pectinolítica. *Pichia fermentans* produziu altas concentrações de ésteres, enquanto *Saccharomyces* sp. apresentou alta atividade pectinolítica. Portanto, a inoculação destas leveduras foi promissora para fermentação pela via úmida, pois além das características citadas, a bebida do café apresentou aromas frutados e de manteiga.

Bactérias ácido láticas (BAL) também apresentam potencial como culturas iniciadoras em café fermentado pela via úmida. Cepas de *Lactobacillus plantarum* se

mostraram capazes de produzir ácidos orgânicos e ésteres que podem aprimorar a qualidade sensorial do café. O tempo de fermentação neste experimento foi acelerado pelo processo de inoculação, reduzindo de 24 para 12 h (PEREIRA et al., 2016). Outro papel executado pelas bactérias do ácido lático é o efeito antagonista contra cepas de fungos ocratoxigênicos como *Aspergillus westerdijkiae* por cepas de *Lactobacillus brevis* (PEREIRA et al., 2016).

O efeito da fermentação pela via semi-seca de grãos de café inoculados com fungo filamentoso e a interferência no perfil de compostos voláteis e não voláteis foi verificado por Lee et al. (2016a, b). Os autores concluíram que a fermentação utilizando *Rhizopus oligosporus* induziu a mudanças positivas e significativas na composição de aromas em café verde e torrado.

Há ainda outras funções que podem ser desempenhadas por microrganismos utilizados como culturas iniciadoras para produção de produtos de valor agregado. Aaplicação de *Bacillus coagulans* como cultura iniciadora em resíduo lignocelulósico de polpa de café para produção de L (+) ácido lático (PLEISSNER et al., 2016; NEU et al., 2016) e a introdução de actinobactérias (*Streptomyces costaricanus* e *Streptomyces exfoliatus*) com atividade celulolítica e xilanolítica para acelerar a degradação de compostos lignocelulósicos aumentando a produtividade de compostos bioativos como polifenóis, antocianinas, taninos e catequinas (KURNIAWATI et al., 2016).

O uso de culturas iniciadoras na fermentação do café é uma alternativa economicamente viável para obter um café diferenciado, agregando valor ao produto, ajudando a manter controle sobre a fermentação e padronizando o processo fermentativo (EVANGELISTA et al., 2014a, b; PEREIRA et al., 2014).

2.8 MALDI-TOF (Matrix Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry)

Vários métodos fisiológicos, sorológicos, bioquímicos e genômicos como o sequenciamento do gene 16S rRNA são rotineiramente utilizados para identificação de bactérias. No entanto, novas tecnologias para rápida identificação de bactérias são essenciais em diferentes campos da microbiologia (De BRUYNE et al.,2011). Alguns destes métodos moleculares mais recentes têm a capacidade de identificar o microrganismo ao nível de linhagem e apresentam, quando comparados com os métodos convencionais, alto nível de precisão e confiabilidade. Todavia, os métodos

baseados na biologia molecular, além de necessitarem de pesquisadores altamente especializados, são métodos que necessitam de reagentes dispendiosos e específicos (PADLIYA, COOPER, 2006; SANTOS et al., 2012).

MALDI-TOF MS (*Matrix-assisted laser desorption/ionization time-of-flight Mass Spectometry*) é uma técnica de espectrometria de massas de alta resolução amplamente utilizada para determinar massas moleculares de compostos. Essa técnica foi desenvolvida por Karas et al. (1985, 1987) e Tanaka et al. (1988) para investigar a espectrometria de massa de compostos orgânicos de alto peso molecular através de ionização suave das moléculas resultando em mínima fragmentação e resultando em espectros de proteínas como biomarcadores (RODRIGUES et al., 2011; TANAKA et al., 1988).

Diferentes fatores experimentais que incluem o preparo da amostra, lise do material celular, soluções para matriz e solventes orgânicos afetam a qualidade e reprodutibilidade da técnica (De BRUYNE et al., 2011). A composição química celular dos microrganismos pode ser analisada, gerando rapidamente um *fingerprint* molecular para identificação microbiana. A excelente reprodutibilidade desta técnica baseia-se na análise das proteínas ribossomais que são expressas de forma constante e existem em abundância dentro das células. A gama de massa molecular usada para estas análises é determinada entre 2 a 20kDa, onde as referidas proteínas ribossômicas aparecem. O procedimento é rápido e, em alguns casos, a preparação da amostra não necessita de pré-tratamento (SANTOS et al., 2010; RODRIGUES et al. 2011; SANTOS et al., 2011).

A base do MALDI-TOF MS se concentra em três aspectos principais: (i) uma fonte de ionização, onde ocorre a vaporização da amostra através de um laser; (ii) um analisador de massas (time-of-flight) que separa os íons de acordo com a relação massa/carga (m/z); (iii) um detector de partículas para monitorar os íons separados (DEKKER e BRANDA, 2011; TONOLLA et al., 2009).

A identificação realizada por MALDI-TOF, ao nível de gênero, apresenta aproximadamente 60% de identificações corretas. Em contraste, os sistemas de identificação baseados em análises bioquímicas atribuíram 25% de gêneros incorretos. Essa diferença é ainda mais evidente ao nível de espécie, portanto a técnica de MALDI-TOF é mais eficiente em relação a identificações rotineiramente feitas em laboratório (URWYLER; GLAUBITZ, 2016).

A técnica tem sido utilizada para rápida identificação de microrganismos patogênicos de ordem clínica (LIMA-NETO et al., 2014; NOMURA, 2015), bactérias anaeróbicas (BARBA et al., 2014; HSU et al., 2014), Lactobacilos de diversas origens (DEC et al., 2014), bactérias acéticas (TRCEK; BARJA, 2015; ANDRÉS-BARRAO et al., 2013), comunidades bacterianas em bebidas fermentadas (LV et al., 2016), bactérias ácido láticas em queijos (NACEF et al., 2016) e diversidade microbiana na fermentação de cacau (SCHWENNINGER et al., 2016).

2.9Análise sensorial do café

O café é um produtomuito valorizado com base em parâmetros qualitativos, em que, quanto melhor a qualidade, maiores os preços a serem obtidos. A qualidade depende de diversos fatores que estão presentes em todas as etapas da produção até o preparo da bebida (MORALES, 1989). Os métodos para avaliar a qualidade do café estão intimamente ligados aos aspectos físicos do grão e em características sensoriais.

A química do café é altamente complexa e interfere diretamente na qualidade da bebida. Durante o processo de torrefação são gerados inúmeros compostos voláteis que impactam no aroma e sabor da bebida (RIBEIRO et al., 2009; FLAMENT, 2002). Algumas pesquisas avaliam a composição química do café e sua qualidade sensorial para compreender a relação entre estas características (RIBEIRO et al., 2009, 2011; BHUMIRATANA et al., 2011; RENDÓN et al., 2014; TOCI e FARAH, 2014; WEI et al., 2014).

Normalmente, a qualidade do café é avaliada por critérios como tamanho do grão, cor, formato, método de processamento, ano de colheita, presença de defeitos e análise sensorial (BANKS et al., 1999). Dentre estes, analise sensorial e presença de defeitos são os mais empregados como critério de classificação (FRANCA et al., 2005a). A Instrução Normativa nº8, de 11 de junho de 2003, do Ministério da Agricultura, Pecuária e Abastecimento, foi instituída no Brasil e estabelece padrões de classificação do café beneficiado Grão Cru (BRASIL, 2003). A classificação da bebida do café também é feita por uma análise sensorial conhecida como "prova de xícara". Atualmente esta técnica é reconhecida mundialmente pela *Specialty Coffee Association of America* (SCAA). A SCCA foi fundada em 1982 por um pequeno grupo de profissionais da área do café, visando um fórum comum para discutir questões sobre qualidade e padrões para o comércio de cafés especiais. A SCAA representa nos dias

atuais a maior associação de café do mundo, com cerca de 2500 membros (SCAA, 2016).

The Coffe Cupper's Handbook foi criando em 1985 por Ted Lingle, ex-diretor executivo da SCAA e atual diretor executivo do Coffee Quality Institute para estabelecer critérios de preparo e avaliação do café para prova de xícara. Os padrões podem ser ótimas ferramentas para a indústria do café, pois são instrumentos de referência confiáveis estabelecidos por especialistas em café. Os especialistas estabelecem medidas quantificáveis e qualificáveis, baseada em testes científicos, que estabelecem valores e/ou intervalos de valores para cada atributo avaliado. Atualmente, o SCAA tem padrões para água, café verde, torrefação, preparo do café e a prova de xícara. Dentre os critérios avaliados na prova de xícara estão atributos como flavor, aroma, corpo, gosto residual, acidez, uniformidade, doçura entre outros (SCCA, 2016).

Em combinação com técnicas tradicionais como a prova de xícara, a *Temporal Dominance of Sensations* (TDS) é uma técnica de análise sensorial que avalia a evolução de sensações percebidas como dominantes durante certo período de tempo. Esta técnica tem sido amplamente utilizada em análise de alimentos, pois confere uma abordagem sobre as sequências em que as sensações são percebidas como dominantes ao longo de um tempo determinado (PINEAU et al., 2009; 2012). A análise de TDS não requer treinamento e diversos atributos podem ser avaliados simultaneamente (Di MONACO, et al., 2014).

TDS tem se mostrado uma técnica promissora para análise sensorial de café. Barron et al. (2012) avaliaram o impacto do creme do café espresso na percepção de aroma. Os autores relataram que a técnica de TDS fornece uma eficiente ferramenta para distinção de cafés e que a presença de espuma no café estava associada ao atributo "torrado". Dinella et al. (2013) relataram o uso de TDS para café adoçado com três diferentes concentrações. Evangelista et al. (2014a, b; 2015) utilizou a técnica de TDS para avaliar cafés inoculados com culturas iniciadoras, processados pela via seca e semi-seca e também pela fermentação espontânea na via úmida. Portanto, a técnica de TDS provê instrumentos necessários para análise sensorial de café e quando comparada a outras técnicas se torna ainda mais informativa.

3 CONSIDERAÇÕES FINAIS

O uso de leveduras como culturas iniciadoras em café processado pela via semi-seca é capaz de melhorar sensorialmente a bebida do café. As leveduras *Saccharomyces cerevisiae*tem grande potencial para serem utilizadas como culturas iniciadoras em café.

É de extrema importância o conhecimento da microbiota do café e o impacto que pode causar na qualidade. Com o uso de técnicas de espectrometria de massas como MALDI-TOF é possível identificar microrganismos presentes na fermentação do café. Através do estudo das diferenças microbiológicasem café é possível dispor informações acerca de prováveis inoculantes para os diversos processamentos do café. As informações obtidas sobre composição química do café verde e torrado podem auxiliar na escolha de uma variedade mais indicada para cada tipo de fermentação. Por fim, a análise sensorial dos cafés utilizando duas técnicas (prova de xícara e Temporal Dominance of Sensations) melhora a descrição das características sensoriais das amostras.

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SEGUNDA PARTE

Artigos científicos

ARTIGO 1- Controlled fermentation of arabica coffee using starter cultures: a sensory perspective

Artigo Publicado no periódico:*LWT -Food Science and Technology*

ABSTRACT

Sensory analysis is one of the most important techniques to assess coffee quality. This study aimed to evaluate the sensory effect of inoculatingtwo varieties of coffee (Ouro Amarelo and Mundo Novo) processed by a semi-dry method,with three yeast strains (*Saccharomyces cerevisiae* CCMA 0200 and CCMA 0543 and *Torulaspora delbrueckii* CCMA 0684, respectively) of Culture Collection of Agriculture Microbiology (CCMA). Two sensory analysis techniques were used (cup taste and temporal dominance of sensations analysis, TDS). Pulped coffee beans were inoculated with the respectively yeast strains and compared to a non-inoculated sample. Ouro Amarelo showed the highest scores for the attributes evaluated compared to variety Mundo Novo. The use of strains CCMA 0543 and CCMA 0684 improved the beverage sensations of both coffee varieties. Variety Ouro Amarelo - CCMA 0543, highlighted acidity and nuts sensations and Mundo Novo - CCMA 0543 and CCMA 0684 treatments reduced the astringency sensation. The addition of CCMA 0543 starter culture highlighted the acidity of the coffee showing better sensory results among the yeast strains, for both coffee varieties. The use of two sensory analysis techniques provided better descriptive and comparative analysis of the sensory characteristics of the treatments.

Keywords: coffee; sensory analysis; starter culture; temporal dominancesensations

1 Introduction

Coffee is one of the most important worldwide commodities and is internationally marketed as green coffee. About sixty tropical and subtropical countries produce coffee as their main export product (FAOSTAT, 2016; Lashermes, Andrade,

&Etienne, 2008; Sakiyama & Ferrão, 2015; Vieira, 2008). Minas Gerais State is the largest coffee producer in Brazil. It is well-known for producing high-quality coffees with a great diversity of flavor and aroma. The characteristics of each coffee are unique to the production area, mainly due to variations in climate, latitude, altitude and production system (Alves, Volpato, Vieira, Borém, & Barbosa, 2011). The quality of coffee has been valued in recent years, giving credibility to specialty coffees, which are characterized by a set of aromas and balanced memorable flavors and the absence of defects. These attributes are associated with the coffee origin and the cultivated genotype (Taveira et al., 2014).

Most of the coffee beverages prepared in the world are produced from the specie *Coffea arabica*, which is considered superior due to its highly appreciated sensory properties (Bertrand, Guyot, Anthony, & Lashermes, 2003; ICO, 2016; Lashermes, Bertrand, & Etienne, 2009), consequently reaching high prices on the export markets (Gielissen & Graafland, 2009).

The technologies that improve the quality of coffee can be implemented at different stages of production, from the coffee plantation up to the storage. The processing is intended to ferment and dry the coffee cherries. The presence of different microorganisms during coffee fermentation interfere with the final characteristics of the beverage. Thus, research has been developed to control this fermentation and improve the quality using starter cultures (Evangelista, Miguel, Silva, Pinheiro, & Schwan, 2014a; Evangelista et al., 2014b; Pereira et al., 2014, 2015; Puerta-Quintero & Molina, 2015; Silva et al., 2013).

The sensory properties of coffee have been the focus of studies for many years (Morale, 1989; Sunarharum, Williams, & Smyth, 2014). Usually, evaluation of coffee sensory properties are conducted by human tasters, specifically trained for this purpose

(Teixeira, Teixeira, & Brando, 2005). Cupping, also known as the cup test, is a systematic method that assesses the aroma and taste of coffee and is commonly used by growers, buyers, and roasters to analyze the quality and flavor profile of coffee. One of the standards often used to evaluate specialty coffees is proposed by Specialty Coffee Association of America (SCAA) and involves training panelists who evaluate the coffee from green through to roasted (Sunarharum, William, & Smyth, 2014, SCAA, 2016).

The temporal dominance of sensations (TDS) technique was developed at the "Centre Européen des Sciences du Goût" in the LIRIS lab in 1999 (Di Monaco, Su, Masi, & Cavella, 2014) and was first presented at the Pangborn Symposium by Pineau, Cordelle, and Schlich (2003). The TDS system analyzes several sensory attributes simultaneously over time and determines the dominant sensation or prevalence of one sensation over the others during product evaluation (Di Monaco, Su, Masi, & Cavella, 2014; Le Révérend, Hidrio, Fernandes, & Aubry, 2008; Pineau et al., 2009; Pineau et al., 2012). The TDS curves are constructed through statistical calculations. This technique has been used to evaluate various food products, such as wines and beers (Ishikawa & Noble,1995; King & Duineveld, 1999;), breakfast cereals (Lenfant, Loret, Pineau, Hartmann, & Martin, 2009), chocolate (Rodrigues, Condino, Pinheiro, & Nunes, 2016).Coffee presents changes in sensorial perception over time, thus is advantageous use of TDS that showed in sequence the sensations perceived at the time of tasting, interesting to complement the cup testtraditionally used in this food (Dinnella, Masi, Naes, & Monteleone, 2013; Evangelista et al., 2014a, 2014b; Evangelista, Miguel, Silva, Pinheiro, & Schwan, 2015).

Quantitative Descriptive Analysis (QDA) is one of the most common analysis used which enables complete sensory description of the analyzed product (Stone & Sidel, 2004). This method can be used to various types of food, to evaluate different

sensory properties as odour, colour, texture and flavor, presented graphically (Rogalski , Nowak& Szterk, 2016; Szterk & Jesionkowska , 2015). The TDS analysis on the other hand enables to evaluate different sensorial attributes over time. It is interesting to use when is desired evaluate on product sensorial changes perceived during the eating and drinking process.

The objective of this work was to use two sensory techniques,cup test and TDS analysisto evaluate the effect of inoculating two coffee varieties [Ouro Amarelo (OA) and Mundo Novo (MN)] produced by the semi-dry method,with three distinct starter cultures, *Saccharomyces cerevisiae* CCMA 0543 and CCMA 0200 and *Torulaspora delbrueckii* CCMA 0684, respectively.

2 Material and methods

2.1 Microorganisms used as start cultures

The microorganisms used as starter cultures were from the Culture Collection of Agricultural Microbiology (CCMA), Federal University of Lavras, Lavras, Minas Gerais, Brazil. The fermentations were performed with *S. cerevisiae* CCMA 0543 and CCMA 0200, and *T. delbrueckii* CCMA 0684 yeasts. The CCMA 0543 and CCMA 0684 microorganisms had been previously isolated from coffee fruit (*C. arabica* L. var. Acaiá) during dry and semi-dry fermentative processes (Silva, Schwan, Dias, & Wheals, 2000; Vilela, Pereira, Silva, Batista, & Schwan, 2010). *S. cerevisiae* CCMA 0543 has been previously tested as a potential starter culture in coffee fermentation (Silva et al., 2013; Evangelista et al., 2014a, 2014b).

The isolates were stored at -80 ºC and were reactivated in YEPG tubes containing 9 mL of liquid medium [glucose 20 $g.L^{-1}$(Merck), yeast extract 10 $g.L^{-1}$(Merck) andbacteriological peptone 10 $g.L^{-1}$(Himedia), pH 3.5]. The cultures were incubated at 28 ºC for 48 h, and then transferred to 90 mL of YEPG and incubated at 28 ºC, 150 rpm for 24 h. The yeast cells were transferred to larger volumes of YEPG until a concentration necessary to inoculate the coffee at 10^7 cells/g was reached.

2.2 Harvest and coffee processing

Coffee fruits (*C. arabica* L.) were collected mechanically at peak harvest (June 2014) on a farm in Patrocínio, Minas Gerais, Brazil (970–1200 m above sea level). Fruits of varieties OA and MN at maximum maturation were used to perform the experiment. The coffee fruit was pulped in a horizontal machine (Ecoflex, Pinhalense, São Paulo, Brazil). The coffee mucilage was not removed.

The pulped coffee (60 kg) was spread on a suspended terrace. Treatments were performed by inoculating each coffee variety separately with one yeast strain. The following treatments were realized: *S. cerevisiae* CCMA 0200 (OA-0200), *S. cerevisiae* CCMA 0543 (OA-0543) and *T. delbrueckii* CCMA 0684 (OA-0684) inoculated in variety OA and control without inoculation (OA-control), in addition to*S. cerevisiae* CCMA 0200 (MN-0200), *S. cerevisiae* CCMA 0543 (MN-0543) and *T. delbrueckii* CCMA 0684 (MN-0684) inoculated in variety MN and without inoculation (MN-control) (Figure 1). The treatments were performed in duplicate. The pulped coffee remained on the suspended terraces until they reached 11–12% (w.b.) moisture content.

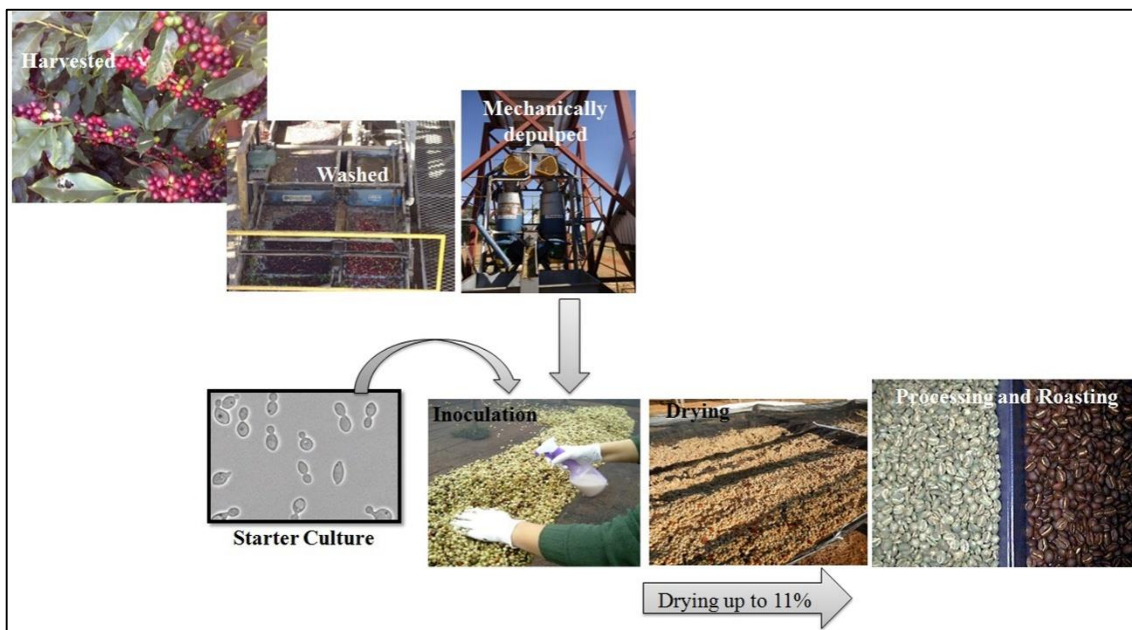

Fig. 1. Flowchart process and inoculation of yeast.

2.3 Sensory analysis

2.3.1 Sensory analysis based on the SCAA protocol

The sensory analysis was performed by three certified specialty coffee judges, for coffee brewing and roasting (Lingle, 2011). The coffee was roasted to a level corresponding to 58 points for whole beans and 63 points for ground beans, with a tolerance of ± 1 point. One hundred grams of beans from each sample were roasted.

For the sensory evaluation, five cups of each sample were tasted, with one session of sensory analysis for each repetition. The evaluated sensory attributes were grouped into “subjective” and “objective” categories. The “subjective” attributes were fragrance/aroma, flavor, acidity, body, balance (synergistic combination of flavor, aftertaste, acidity, and body;complement or contrast to each other), aftertaste and overall impression (taste experience of the evaluator individually, personal appraisal). They

were scored according to their quality on a scale of 6−10 points with 0.25 point increments. The “objective” category included uniformity, sweetness, and clean cup (i.e. absence of defects). The objective attributes were scored on a scale from 0−10 points, with 2 points awarded for each cup that presented satisfactory levels of each attribute. The sum of all these evaluated attributes is the total score. Besides these evaluation the tasters were also asked to describe the characteristic flavors of each coffee.

2.3.2 TDS

2.3.2.1 Panelist selection and training

TDS analysis was conducted according to Pineau et al. (2009). Each treatment was examined in duplicate under the same conditions, totaling 16 evaluations per taster, using the data acquisition program SensoMaker version 1.8 (Nunes & Pinheiro, 2012). The analysis was performed by selected tasters, and the panelists were selected through a triangular test comprising 50 people. Three samples were presented to the tasters, among which two samples were the same and one sample was different. The tasters were asked to identify the different sample. This test was performed with two forms of processed coffee (pulped coffee and natural coffee). Eleven coffee consumers (five females and six males), aged 20–30 years were selected. These panelists underwent sessions to allow familiarization with the sensations evaluated in coffee (Table 1). The panelists were also introduced to various concepts of sensory analysis that included "sequence" (the succession of different sensations in the mouth), "dominance" (the feeling that causes more attention at any given time, the dominant sensation is not

necessarily the one with the greatest intensity), and "weak but dominant" (a sensation may be perceived weaker but be dominant) (Charles et al., 2015; Pineau et al., 2009). The tasters were trained to use the software (SensoMaker, version 1.8) and procedures for data acquisition.

Table 1. References used to familiarize the selected panel with sensations involved in the temporal dominance of sensations test of coffee.

Sensations	References
Sweet taste	Coffee sweetened with fructose (5 g/L)
Bitter chocolate taste	Cocoa liquor
Nutty flavor	Coffee-flavored nuts and nuts
Astringent sensation	Coffee added with green beans
Acid taste	Coffee with citric acid (18.75 mL/L)
Fruity flavor	Coffee flavored fruity and dried fruits
Bitter taste	Coffee dark roasts

2.3.2.2 Data acquisition and statistical analysis

The sensations identified during the analyses performed according to thecupping test were selected for further TDS evaluations. Sweetness, bitter chocolate, nut, astringent, acid, fruity and bitter were selected as the sensations for the coffee studies performed by the panelists (Q Grader).

The tests were performed in closed cabins with white illumination, at the Sensory Analysis Laboratory, Food Science Department, Federal University of Lavras (Lavras, Minas Gerais, Brazil). The beverage from each sample was prepared in the same manner as the cupping analysis. Individual samples of 20 mL were randomly presented to the panelists in plastic cups of 50 mL, at 50 °C. The panelists imbibed the coffee and moved it around in their mouths for 5 s, before swallowing it and starting the evaluation. The panelists entered the sensation experienced into a computer until no sensation was perceived after a maximum of 30 s. After each sample, the subjects rinsed their mouth with water.

Data collection began upon tasting and consisted of selecting the most dominant flavor profile characterization of the coffee perceived at a given time using the SensoMaker software (Nunes & Pinheiro, 2012). TDS curves were plotted, showing the percentage of subjects that selected the sensation as dominant at a specific time (i.e. the dominance rate) (Pineau et al., 2009).

Quantitative parameters can be used to summarize the TDS curves. Among them, is the maximum dominance rate (M). which corresponds to the higher value dominance rate shown by a sensation compared to the other sensations. This parameter was obtained and analyzed by principal component analysis (PCA) using SensoMaker software (Nunes & Pinheiro, 2012), to evaluate differences and similarities among the samples and their characteristics. An $n \times m$ matrix was built with n lines(samples) and $m$columns (parameters) for each sensation.

The cup quality data were evaluated by analysis of variance (ANOVA) and the Scott-Knott test was used for comparison between means, adopting a significance level of 5% probability. The test was performed considering each treatment in isolation using Sisvar 5.6 software (Ferreira, 2014).

3 Results

3.1 Coffee cupping

The cup quality resultsrevealed the attributes of uniformity, clean cup and sweetness had the maximum notes for all treatments quantified by all the cuppers (10.00). An interaction test performed among the factors (variety of coffee and inoculum) was not significant, indicating that the factors were independent, i.e. the

behavior of one factor was independent of variation (absence or presence) of the other factor (Gomes, 2000). In this instance, the separate analyses for each treatment are valid. For the aroma attribute, there was a significant difference between the treatments performed with the variety OA compared to MN.

For the other attributes showed in table 2 (acidity, aftertaste, balance, overall and final score), besides the difference between OA and MN treatments, where OA obtained the highest score, two treatments were significantly different. OA-0543 treatment attained the highest scores for flavor, acidity, body, aftertaste, balance, overall, and final score (7.50, 7.71, 7.42, 7.46, 7.50, 7.38, and 82.17, respectively) (Table 2) and was significantly different from the other treatments. MN-0200 had the lowest scores for flavor, acidity, body, aftertaste, balance, overall, and final score (6.17, 6.17, 6.33, 6.08, 6.17, 6.08, and 73.75, respectively) and differed from the other treatments.

In general, OA-0543 stood out, showing the highest final score (82.17 points). Coffees inoculated with *S. cerevisiae* CCMA 0200 had the lowest score across all treatments analyzed in this study, with a total score of 79.92 and 73.75 for OA and MN, respectively (Table 2).

The panelists qualitatively described the treatments. OA-control was depicted as chocolate, lemon, nuts and caramel flavor, while OA-0200 was described as having high astringency. The OA-0543 treatment provided a delicate acidity of lemon, caramel and sweet flavor. An acidity associated with an apple flavor but having slight astringency was ascribed to the OA-0684 treatment. Treatment performed with MNshowed some unpleasant characteristics. In particular, MN-control was considered to have a green coffee flavor and high astringency, MN-0200 provided a green flavor that was immature and astringent. MN-0543 treatment was also described as astringent,

however, an aroma of almonds and milk chocolate was reported, while MN-0684 also presented high astringency and bitterness.

Table 2. Scores of attributes obtained from the cup test, for Ouro Amarelo and Mundo Novo coffee varieties.

Samples	Fragrance/ Aroma	Flavor	Acidity	Body	Aftertaste	Balance	Overall	Final Score
OA-control	7.29 ± 0.25a	7.04 ± 0.33b	7.33 ± 0.26b	7.25 ± 0.22a	7.17 ± 0.20b	7.21 ± 0.25b	7.17 ± 0.26b	80.46 ± 1.40b
OA–CCMA 0200	7.25 ± 0.42a	7.17 ± 0.38b	7.17 ± 0.38b	7.21 ± 0.40a	7.04 ± 0.33b	7.13 ±0.21b	6.96 ± 0.25b	79.92 ± 1.96b
OA–CCMA 0543	7.21 ± 0.25a	7.50 ± 0.16a	7.71 ± 0.25a	7.42 ± 0.13a	7.46 ± 0.10a	7.50 ±0.00a	7.38 ± 0.21a	82.17 ± 0.70a
OA-CCMA 0684	7.33 ± 0.38a	7.17 ± 0.30b	7.08 ± 0.13b	7.21 ± 0.25a	7.04 ± 0.25b	7.08 ±0.20b	7.04 ± 0.10b	79.96 ± 0.68b
MN-control	6.67 ± 0.41b	6.50 ± 0.32c	6.58 ± 0.49c	6.75 ± 0.42b	6.58 ± 0.38c	6.58 ±0.38c	6.58 ± 0.38c	76.25 ± 2.16c
MN–CCMA 0200	6.75 ± 0.54b	6.17 ± 0.24c	6.17 ± 0.24d	6.33 ± 0.47c	6.08 ± 0.12d	6.17 ±0.24d	6.08 ± 0.12d	73.75 ± 1.81d
MN–CCMA 0543	6.88 ± 0.31b	6.42 ± 0.26c	6.54 ± 0.10c	6.71 ± 0.25b	6.38 ± 0.21c	6.54 ± 0.33c	6.42 ± 0.26c	75.88 ± 1.50c
MN–CCMA 0684	6.83 ± 0.29b	6.33 ± 0.29c	6.50 ± 0.50c	6.83 ± 0.29b	6.33 ± 0.29c	6.50 ± 0.00c	6.50 ± 0.00c	75.83 ± 0.76c

OA: Ouro Amarelo variety. MN: Mundo Novo variety. Data are presented as the mean ± standard deviation. Means followed by the same letter in the column do not differ significantly by the Scott-Knott test ($P > 0.05$).

3.2 TDS

The TDS analysis provided the dominant sensations in the coffee beverages. The TDS curves for the OA and MN treatments are presented in Figures 2 and 3, respectively.

For OA-control treatment, the main sensations perceived were acid (from 7−13 s of analysis), followed by bitter (from 11−16 s of analysis and again at 29 s) (Fig. 2A). In contrast, OA-0200 presented astringent as the first dominant sensation (from 10–13 s), followed by bitter chocolate (from 25−29 s) (Fig. 2B). OA-0543 revealed the acid sensation was initially dominant (from 8−16 s), followed by nut for a short period at the end of analysis (from 29−30 s) (Fig. 2C). Acid (from 10−19 s) then astringent (11−23 s) were the initial sensations to dominate in OA-0684, followed by bitter (24–30 s) (Fig. 2D). The acid sensation predominated in treatments with the variety OA. Furthermore, the inoculated treatments showed sensations that were not observed in the control (nuts for OA-0543, astringent for OA-0684, and astringent and bitter chocolate for OA-0200). The sensation of bitterness was predominant in all treatments with the variety MN, as shown in Figure 3. The MN-control treatment presented bitter as the first dominant sensation (remained until 12 s). Between 20–30 s, the bitter sensation dominated again. At 26 s of the analysis, astringency was the governing sensation (Fig. 3A). The MN-0200 treatment also showed astringent as thedominant sensation (between 5−15 s), followed by bitter, which was perceived between 10–20 s and after 25 s (Fig. 3B). The MN-0543 treatment was dominated by a bitter sensation that started at 5 s and remained throughout the analysis (Fig. 3C). MN-0684 had an overwhelming bitter sensation after 20 s of analysis that remained until the analysis ceased (Fig. 3D).

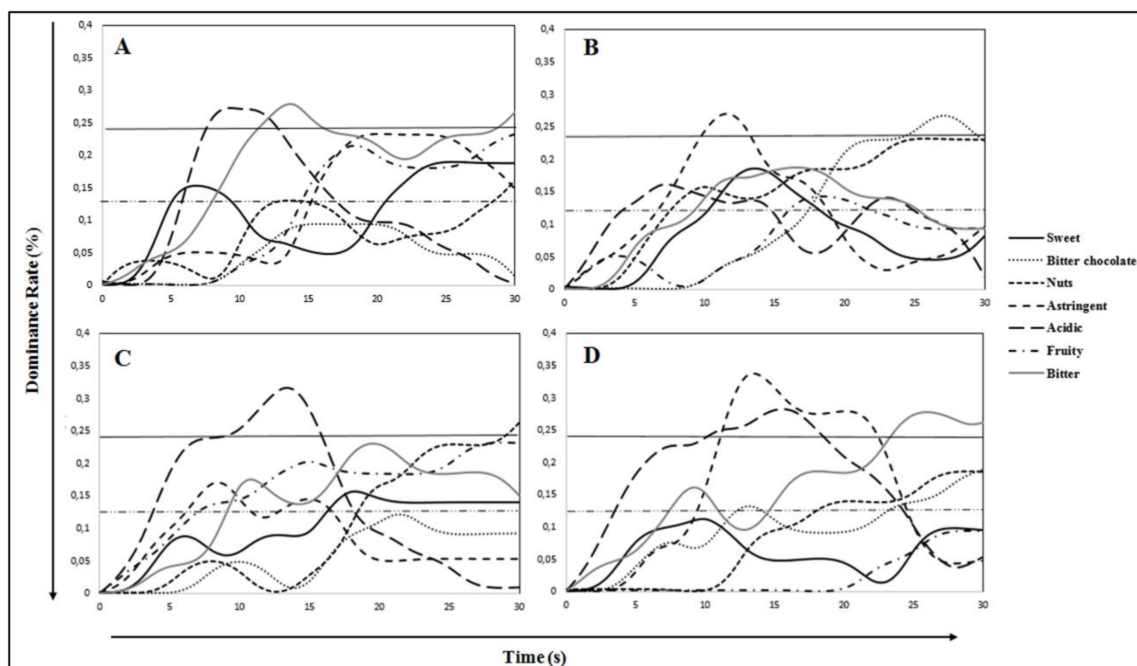

Figure 2. Temporal dominance of sensations curves of coffee variety Ouro Amarelo. A) control (without inoculation), B) inoculated with *Saccharomyces cerevisiae* CCMA 0200, C) inoculated with *S. cerevisiae* CCMA 0543, D) inoculated with *Torulaspora delbrueckii* CCMA 0684.

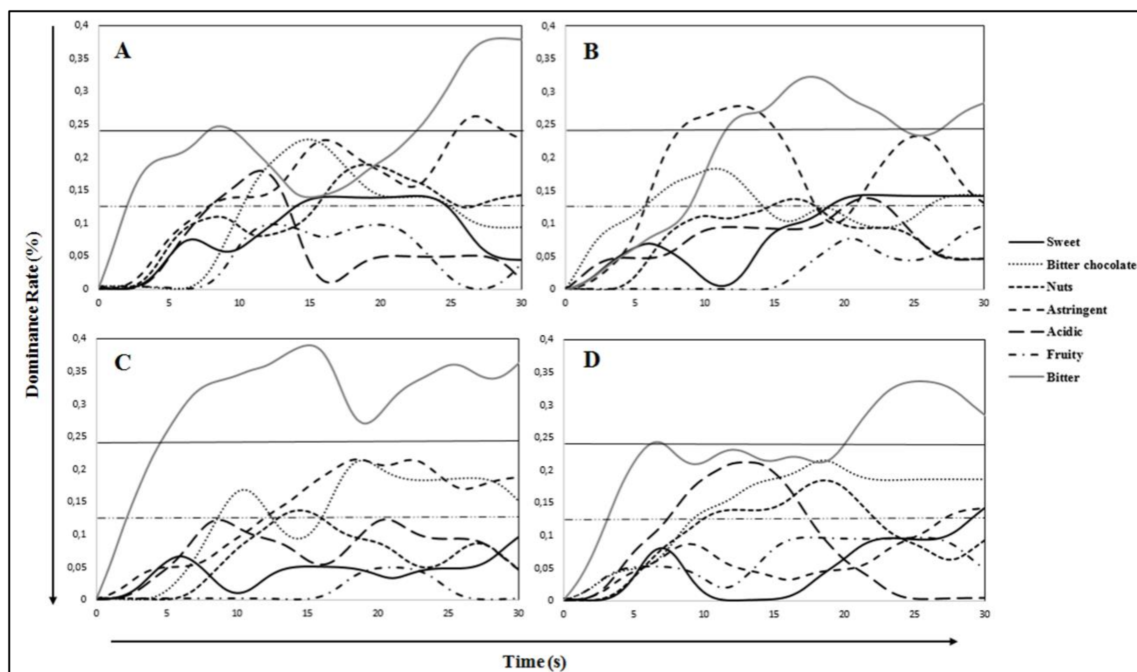

Figure 3. Temporal dominance of sensations curves of coffee variety Mundo Novo. A) control (without inoculation), B) inoculated with *Saccharomyces cerevisiae* CCMA 0200, C) inoculated with *S. cerevisiae* CCMA 0543, D) inoculated with *Torulaspora delbrueckii* CCMA 0684.

The maximum dominance rate (M) is a parameter that can be generated by the TDS analysis. PCA analysis was performed for this parameter for both coffee varieties, as shown in Figure 4. The two principal components accounted for 71.28% of the total variability in the data set (PC1 51.84% and PC2 19.44% of the total variance), showing discrimination between samples. The OA-control and OA-0543 were mainly characterized by a higher maximum dominance rate for acid and fruity, respectively. In the TDS curves, fruity was not a dominant sensation for the OA-0543 treatment, but was close to the significance threshold, implying that it had a higher chanceof being perceived by the tasters. Acid was a dominant attribute in the OA-control treatment. OA-0200 was characterized by a higher maximum dominance rate for nut and bitter chocolate, while OA-0684 was mainly characterized by a higher maximum dominance rate for astringent. Treatments of the MN variety were grouped together and mainly characterized by a maximum dominance rate of astringent and bitter. These characteristics were also observed in the TDS curves.

4 Discussion

In this study, two methods were used to assess the sensory characteristics of pulped coffee varieties OA and MN using three different yeast strains as starter cultures. One method was the cupping test, which was performed according to the traditional SCAA method (Table 2), while TDS was the second technique (Fig. 2 and 3).

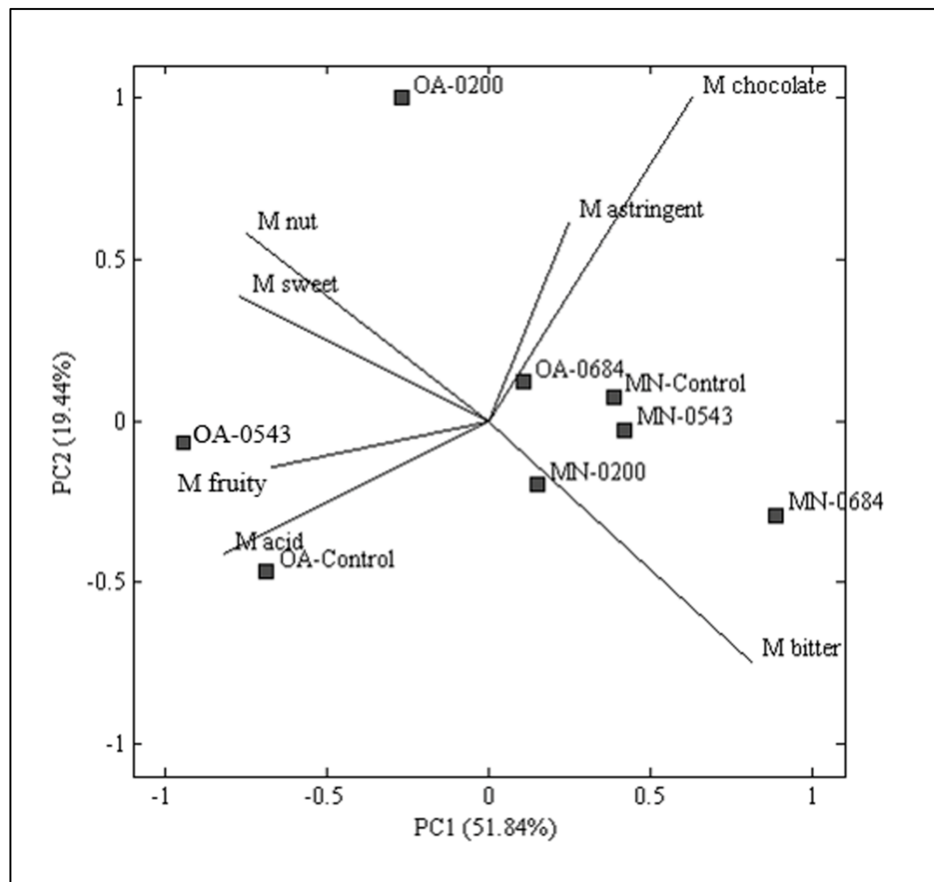

Figure 4. Principal component analysis loading plot for maximum dominance rate (M) parameter generated from the temporal dominance of sensations curves of Ouro Amarelo and Mundo Novo coffee varieties.

The sensory analysis results of the cup test demonstrated that treatments OA-control and OA-0543 could be considered as providing specialty coffee. According to the SCAA, coffee beverages that receive scores above 80 can be considered specialty coffee. For these coffees, each step, from choosing the coffee cherries and location, to harvesting, fermentation, drying, storage, roasting (Masi, Dinnella, Barnabà, Navarini, & Monteleone, 2013) and preparation of the beverage, is handled with the best possible care and knowledge. The steps operate in harmony to maintain standards and excellence from start to finish (SCAA, 2016). Treatments with the variety MN showed a very good coffee but could not be considered as a specialty because its highest score was below 80 (Table 2).

For the treatments with OA, the TDS sensory analysis complemented the cupping test results because the selected tasters perceived the dominant sensations, such as acid, bitter, astringency, bitter chocolate and nuts. The SCAA trained tasters also reported these same attributes. OA-0200 and OA-0684 presented pronounced astringency, coinciding with the sensations perceived in the TDS analysis. For treatments with the MN variety, only the astringent and bitter taste dominated. These sensations were also associated with green and immature coffee by the trained tasters, which are characteristics that reveal an astringent taste. In general, the variety OA showed better sensory quality than MN.

The *S. cerevisiae* CCMA 0543 strain provided the best results among the strains evaluated. This yeast might have improved the attributes of flavor, acidity, aftertaste, balance and the overall impression that were responsible for obtaining the highest score among the treatments in the cupping test (Table 2). The treatments with *S. cerevisiae* CCMA 0543 also provided the nuts sensation, which is desirable in a coffee beverage.

The yeast behavior was different among the treatments with MN and OA. The MN coffee inoculated with yeast was not significantly different from the control in the cupping test. MN-0200 provided an astringency in the coffee, which was dominant and undesirable. Consequently, this treatment showed the lowest score in the cupping test. This result was expected because CCMA 0200 was isolated from sugar cane fermentation, and therefore, not suitable for MN coffee. From our results, it was possible to conclude that there is a specificity between the yeast and coffee variety.

The astringency was not perceived in samples inoculated with either *S. cerevisiae* CCMA 0543 or *T. delbrueckii* CCMA 0684 cultures, suggesting that even the TDS notes were comparable to the control, although it was still possible to improve the sensorial taste. These samples presented almond and milk chocolate flavors. *S. cerevisiae* CCMA 0543 and *T.*

delbrueckii CCMA 0684 were isolated during natural coffee fermentation (Silva et al., 2000), which might indicate that these strains are better adapted to the environmental coffee production conditions. In addition to showing variable behavior toward the same coffee variety, the behavior of yeasts may also vary according to the processing conditions used (Evangelista et al., 2014a; 2014b).

Consumers are constantly searching for good quality coffees. Thus, understanding of the coffee microbiota and starter cultures contributes to improving the sensory quality of coffee beverages. The present study demonstrated the sensory differences that exist between coffee varieties (Table 2 and Fig. 3 and 4) and between the yeasts strains used as starter cultures. The variety of coffee had a large influence on the response the yeast presented, exercising a predominant impact on the coffee quality. In contrast, Bhumiratana, Adhikari, & Chambers IV (2011), claimed that the aromatic profile generated in the coffee beverage is influenced mainly by the preparation steps and the degree of roasting than the variety.

Although the TDS technique has been used previously to successfully analyze coffees, studies claim that a combination of methods is important to find potential aromas markers (Charles et al., 2015; Dinnella et al., 2013; Evangelista et al., 2014a, 2014b). In this study, there was a consistency between the cupping test and TDS analysis. The MN variety showed a higher dominance of attributes, such as bitterness and astringency, and had lower overall scores in the cup test. Meanwhile, the OA variety presented acidity, bitterness, chocolate and a predominant nuts flavor that contributed to OA-0543 presenting the highest score among the various treatments studied. Thus, the variety OA was more responsive to treatment with the yeasts.

5 Conclusion

Using starter cultures in coffee fermented by a semi-dry process, the variety OA showed better sensory characteristics than MN after inoculation. Inoculation with *S. cerevisiae* CCMA 0543 produced coffee with higher quality attributes than *S. cerevisiae* CCMA 0200 or *T. delbrueckii* CCMA 0684. The NM variety inoculated with *S. cerevisiae* CCMA 0543 or *T. delbrueckii* CCMA 0684 exhibited reduced astringency. However, *S. cerevisiae* CCMA 0200 did not contribute to enhanced notes in the MN variety. The addition of starter cultures aid to control the fermentation process, thus, ensuring the formation of desirable aromas and flavors, which increase the possibility of producing specialty coffees. The use of two or more sensory analysis techniques is important for the authentication of the results. The cupping test is a standard method for sensory analysis of coffee, while the TDS technique confirmed the cup test findings and allowed improved discrimination of the results.

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ARTIGO 2- Microbiological and chemical-sensory characteristics of three coffee varieties processed by wet fermentation

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ABSTRACT

This work evaluated the bacterial diversity during coffee wet fermentation of the three coffee varieties - Mundo Novo (MN), Ouro Amarelo (OA), and Catuaí Vermelho (CV). Isolates were identified by polyphasic aproach: biochemical tests, Matrix Assisted Laser Desorption/Ionization – Time of Flight (MALDI-TOF) and DNA sequencing. Chemical compositions of coffee beans were determined by high (HPLC) and gas chromatography–mass spectrometry (GC-MS). Thirty-six species of mesophilic bacteria and six lactic acid bacteria were identified. *Lactobacillus plantarum* and *Leuconostoc mesenteroides* were often found in all varieties. Citric acid was the acid detected in higher concentrations. The volatile profile of the green coffee beans changed during the fermentation in the tank, but more significantly, during the roasting process. These volatiles belonged to the classes of acids, alcohols, aldehydes, hydrocarbons. Temporal Dominance of Sensations analysis showed sensorial sensations of acidity (OA and CV), bitterness, chocolate, nuts (MN) and sweetness (CV). The characteristics of each coffee variety were distinct, mainly in relation to total bacteria population, volatile compounds and sensorial profile. The dominant strains might be used for starter cultures. Polyphasic methodology was usufull to bacterial identification. Chemical and sensory analyzes contribute for a better knowledge of coffee fermentation. Our findings are relevant to select starter bacteria for coffee processing to improve and quality standardize.

Keywords: coffee, fermentation, bacteria, sensorial analysis, volatile compounds

Introduction

Worldwide coffee sales continue to increase each year, in part due to increased consumption in emerging markets like Turkey and Russia. In traditional coffee markets, such as the European Union, the United States and Japan, growth is primarily driven by an increased demand for high quality, specialty-grade coffee (ICO 2015). Brazil is the world's largest coffee producer and exporter. Its estimated production for 2018 between 41,74 and 44,55 million of 60 kg bags, with Arabica coffee representing 76% of total production (CONAB 2018). Since the market value depends on the coffee's quality, there is a constant search for improvement not only in production, but also in quality and safety.

Coffee production consists of several steps, including the fermentation and drying that occurs immediately after harvesting. The fermentation process can be performed in three different ways: dry, semi-dry and wet (Brando and Brando 2015). In the wet method, the beans are first separated from the surrounding cherry by pulping and placed in open tanks with water, where they are allowed to ferment for 6 to 72 hours, depending on the environmental temperature, during which the remaining mucilage is degraded and solubilized. The beans are then removed from the tanks and dried in the sun. Epiphytic micro-organisms naturally present in coffee cherries play an important role during fermentation and drying, due to consumption of the pulp and mucilage around the fruit and produce compounds that will directly affect the coffee quality (Silva 2015). Some old studies reported the microorganisms present in the coffee wet fermentation (Agate and Bhat 1966; Avallone et al. 2001; Frank and Cruz 1964; Frank et al. 1965), although the methodology used for identification is outdated. More recently, Evangelista et al.(2015) showed that mesophilic and lactic acid bacteria are the major groups of micro-organisms involved in the wet fermentation process.

The use of appropriate and polyphasic aproach aids the identification of the micro-organisms present throughout the fermentation and will elucidate the diversity and

distribution of species involved in this process. Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) is a technique that can be used to identify micro-organisms. It has been used in studies with different fermented foods, such as vegetables (Nguyen et al. 2013), meat (Doan et al. 2012), silage (Carvalho et al. 2016), kefir vinegar (Viana et al. 2017) and cacao(Schwenninger et al. 2016). In our work, MALDI-TOF MS was used to identify bacteria present during the wet coffee fermentation. In addition, chemical and sensorial analyses of coffee beans were performed to characterize the coffee. The Temporal Dominance of Sensations (TDS) analysis was applied. This analysis has recently been used to describe the sensorial characteristics of coffee, to complement the cup test (Evangelista et al. 2014a, b; Ribeiro et al. 2017b).

The different *Coffea arabica* varieties cultivated in Brazil produce coffee beverages with distinct characteristics. The bacterial profile of these fermentations might be different and needs to be established for each variety, to understand the role of different species or genus in the fermentation process. Ouro Amarelo (OA) and Mundo Novo (MN) varieties have already been studied during semi-dry coffee fermentation and inoculated with yeast. According Ribeiro et al. (2017a) the bean composition of sugars, acids, and volatiles and also sensory response were different in relation to the inoculated coffee variety. In the present study, the chemical and microbiological analyses were carried out to characterize and compare the coffee varieties OA, MN and CV produced through the wet processing.

Materials and methods

Wet fermentation coffee

Coffee cherries of the varieties OA, MN and CV were collected mechanically on a farm located in Patrocínio in Minas Gerais State, Brazil, at 970-1200 meters above sea level.

Coffee cherries were mechanically pulped in a horizontal machine (Ecoflex, Pinhalense, São Paulo, Brazil). Pulped coffee of each variety was fermented separately (4000 Kg) in concrete tanks filled with water. The fermentation was performed twice. The content of each tank was homogenized manually at approximately 4-hour intervals. The complete degradation of the mucilage layer was determined by hand friction between the coffee beans (Velmourougane 2012). Ater fermentation, the coffee was transferred to suspended terraces for sun drying, until it reached 11–12% (w.b.) of moisture, measured using a Moisture Meter G600i (Geaka, São Paulo, Brazil). The following samples (200 g) were taken during processing: i) coffee cherries (CC), ii) pulped coffee at the start of fermentation (PC), iii) coffee without mucilage at end of fermentation (DMC) and iv) dried coffee at the final drying time (DC). It was placed aseptically in sterile plastic bags, and transferred to the laboratory in iceboxes for microbiological analyses. For physicochemical analyses, coffee samples were frozen at -20 °C until analysis. The roasted coffee (RC) was used for sensory and volatile compound analysis.

Proximate composition and pectin

The proximate composition was done in coffee cherries of the varieties studied. The moisture determination was performed according to the method 925.09B (AOAC, 1995) with modifications. The nitrogen content (method 979.09), protein content (method 920.87, using 6.25 as correction factor), fat (method 920.97) and ash (method 923.03), were performed according AOAC(1995). Carbohydrate content was estimated as nitrogen-free extract.

The pectin was performed according to the methodology described by McCready and McComb (1952), using the spectrophotometric determination at 520 nm and total soluble

solids were performed according to Bitter and Muir (1962). The pH was determined by potentiometric analysis.

Microbiological analysis

Quantification, isolation and phenotypic characterization

Aliquots (10 g) of the sample CC, pulped coffee at the start of fermentation (PC), coffee without mucilage at end of fermentation and dried coffee at final drying time were added to flasks with 90 ml of sterile peptone water (in g L^{-1}: 1 bacteriological peptone [Himedia, Mumbai, India]), homogenized in an orbital shaker (280 rpm - 20 min) and used for decimal serial dilution. Mesophilic bacteria were enumerated by spread plating on Nutrient Agar (Himedia, Mumbai, India) and lactic acid bacteria on MRS agar (Merck, Darmstadt, Germany) with added nystatin (Merck) to inhibit growth of yeasts and filamentous fungi. The plates were incubated at 30°C and 35 °C for 72 hours, for mesophilic and lactic acid bacteria, respectively. The morphological characteristics of the colonies (cell size, cell shape, edge, color, and brightness) were recorded and the square root of the number of colonies counted for each morphotype was purified by streaking on new agar plates (Senguna et al. 2009). The phenotypic characterization of the bacterial colonies was performed using Gram staining, catalase and oxidase activities and motility tests (Holt et al. 1994). The pure cultures were stored in an ultra-freezer at -80 °C in the same broth culture media used for plating, containing 20% glycerol (w/w).

MALDI-TOF sample preparation, measurement and data analysis

The isolates obtained (321 colonies) from plating were grown on plates using specific culture medium for each taxonomic group, as described above. The purified cultures were

streaked on new agar plates and incubated at 30°C and 35 °C for 24 and 48 hours for mesophilic and acid lactic bacteria respectively, and then 20 mg of each colony was aseptically transferred to microtubes. The methodology of protein extraction, equipment calibration and method of data analysis was done as described by Carvalho et al. (2016). The equipment used was MALDI-TOF microflex LT spectrometer (Bruker Daltonics, Bremen, Germany).

Molecular identification

Representative strains from each cluster performed by MALDI-TOF and isolates that were not possible be identified using this technique (score < 1.7), were submitted for molecular analyses. A total of 152 isolates were amplified for 16S rRNA analysis, using the primers 27F and 1512R (Devereux and Willis, 1995). The amplified PCR products were sent for sequencing to the Macrogen USA – Humanizing Genomics (Maryland, USA). The sequences were aligned using the CLC Main Workbench 7.7.1 (Quiagen®) sequence alignment editor and were compared to the EzTaxon database server (EzTaxon 2017; Kim et al. 2012) based on 16S rRNA sequence data for the identification of isolates.

Acids and sugars analysis

Organic acids (malic, lactic, acetic, butyric, propionic, citric, oxalic, succinic and tartaric acid) and sugars (fructose, glucose and sucrose) were analyzed.

The following samples were analyzed: pulped coffee at the start of fermentation, coffee without mucilage at end of fermentation and dried coffee at final drying time. Samples were extracted as described by Ribeiro et al. (2017a).

The extracts were analyzed using a HPLC system (Shimadzu, Japan). A Shimpack SCR-101H (7.9 mm x 30 cm) column was used with a 100 mm solution of perchloric acid, with a flow rate of 0.6 ml per min as the mobile phase. The oven temperature was kept at 50 °C for the analysis of acids, detected with a 210 nm UV detector, and at 30 °C for the analysis of sugars, detected with a refractive index detector. The quantification of compounds was performed using calibration curves constructed with different concentrations of standard compounds [malic, propionic and citric acid were purchased from Merck (Germany), lactic, oxalic and tartaric acid was purchased from Sigma-Chemical (EUA), acetic and succinic acids were purchased from Sigma Aldrich (Germany), butyric acid was purchased from Riedel-deHaen (Germany)] and analyzed using the same conditions as for the samples. Analyses were performed twice.

Volatile compounds

The samples analyzed by GC-MS were pulped coffee at the start of fermentation, coffee without mucilage at end of fermentation, dried coffee at final drying time, and roasted coffee (RC). Volatile compounds were extracted using manual headspace-solid phase micro-extraction (HS–SPME).

GC/MS analysis was carried out with a GC-MS-QP2010 from Shimadzu on an Agilent HP-FFAP column (30 m x 0,25 mm x 0,25 µm). The extraction and parameters of analysis were done according to Ribeiro et al. (2017a). Chromatographic and mass spectral data were analyzed with GC-MS solution software from Shimadzu. Compounds were identified by comparison of their mass spectra to the NIST mass spectral library and linear retention index, relative to a series of n-alkanes.

Sensory characteristics analysis

Cupping test

Sensory analysis for coffee roasting and brewing was performed by three certified specialty coffee judges (Q graders), using the methodology proposed by the Specialty Coffee American Association – SCAA (Lingle 2011). The evaluated sensory attributes were fragrance/aroma, flavor, acidity, body, balance, aftertaste, overall impression, uniformity, sweetness, and clean cup. Besides these attributes, tasters were asked to describe the characteristic flavors of each coffee (Ribeiro et al. 2017a).

Temporal Dominance of Sensations (TDS)

The TDS analysis was performed according to Pineau et al. (2009) and under the same conditions as described in Ribeiro et al. (2017a). The analysis was performed by selected tasters, and the panelists were selected through a triangular test comprising 50 people. Three samples were presented to the tasters, among which two samples were the same and one sample was different. The tasters were asked to identify the different sample. This test was performed with two forms of processed coffee (pulped coffee and natural coffee). Eleven coffee consumers (five females and six males), aged 20–30 years were selected. The tasters were trained to use the software (Sensomaker, version 1.8) and procedures for data acquisition.

The tests were performed in closed cabins with white illumination at the Sensory Analysis Laboratory, Food Science Department, Federal University of Lavras (Lavras, MG). The panelists evaluated each sample twice.

Statistical analysis

The data of proximate composition, pectin, and cup quality were evaluated by analysis of variance (ANOVA) and the Scott-Knott test was used for comparison between means, considering each treatment in isolation. A 3 × 3 factorial arrangement of treatment was used to analyze the results of carbohydrates and organic acids: three varieties (Ouro Amarelo Mundo Novo and Catuaí Vermelho and three collected samples (pulped coffee, demucilled coffee and dried coffee). Data were analysed under the following model:

$X = \mu + Var_j + Samp_k + Var \times Samp_{jk} + e_{jk}$

were, μ = global mean; Var_j= varieties effect (j = OA, MN, CV); $Samp_k$ = sample collection time effect (k =PC, DMC and DC); $Var \times Samp_{jk}$ = effect of interaction between varieties and sample collection time; e_{jk} = experimental error.

The tests were performed using Sisvar 5.6 software (Ferreira 2014). Significance level was defined at $p<0.05$ level.

The analysis of the TDS data was made using the Sensomaker Software (Nunes and Pinheiro 2012) and plotting as TDS curves showing the percentage of subjects which selected the attribute as dominant at a specific time (Pineau et al. 2009).

Results

During coffee fermentation, several factors influence the microbiota diversity, metabolites formation and consequently the final quality of coffee. Among these factors, the coffee varieties, fermentation method (solid or submerged fermentation in water, open or closed systems, continuous or sporadic, static or agitated), epiphytic microrganisms, temperature, pH and acidity. The water temperature in the fermentation tank was 21° to 16 °C, while the environmental temperature ranged from 28° to 15ºC and the pH in final of fermentation reached around 4 (begging at 5.5). Fermentation was finished when the complete

degradation of the mucilage layer was tested by friction between the coffee beans. Each variety presented a different fermentation time to complete the process, with 18, 19 and 23 hours for OA, MN and CV varieties, respectively.

Proximate composition and pectin

Proximate composition and pectin content were evaluated in coffee cherries of varieties OA, MN and CV (Table 1). There was significant difference in carbohydrate and soluble pectin contents, among the coffee varieties. The OA and MN varieties presented high carbohydrate content (16.47 and 16.89%), while the OA variety showed high content of soluble pectin (0.33%). The other chemical compounds analyzed were very similar among the varieties. The pulp and mucilage composition were reflected in the normal fermentation time of each variety, and explained why the fermentation time for OA and MN were closer than that for CV.

Table 1. Proximate composition of cherry coffee fruit freshly harvested from the coffee plant variety Ouro Amarelo, Mundo Novo e Catuaí Vermelho in wet weight.

Composition in dry base (%)	**Varieties**		
	Ouro Amarelo	**Mundo Novo**	**Catuaí Vermelho**
Moiture content	63.27a	63.68a	62.64a
Fatty content	1.28a	1.25a	1.40a
Protein content	4.34a	4.72a	3.99a
Ash content	1.71a	1.77a	1.58a
Carbohydrate	16.47a	16.89a	12.39b
Total Pectin	1.10a	2.30a	1.02a
Soluble Pectin	0.33a	0.29b	0.25b
pH	5.03a	4.94a	4.90a

Data are presented as mean. Values with different letters are significant at $p < 0.05$ by Scott–Knott test. Standard deviation of mean ranged from 2.32 to < 0.01.

Microbiological analysis

The bacterial population present in coffee cherry, pulped coffee at beginning of fermentation, coffee without mucilage at end of fermentation and dried coffee at final drying time were quantified by plating (Table 2). The total mesophilic bacteria started with 4.84, 4.69 and 4.10 log CFU per g for OA, MN and CV, but decreased throughout the process until it reached values of 2.48, 2.78 and 2.70 log CFU per g for OA, MN and CV. The largest population of mesophilic and lactic acid bacteria was different in each sample (for each processing step) and coffee variety. The expressive mesophilic bacteria were detected in CC and DMC samples. Similar behavior was observed with lactic acid bacteria (6.15, 5.49 and 3.64 log CFU per g for OA, MN and CV); however, the population was higher than mesophilic bacteria. Most of the population values declined until dried coffee (3.11, 3.75 and 3.96 log CFU per g for OA, MN and CV).

Three hundred and twenty-one bacteria isolates were identified by a combination of MALDI-TOF and sequencing of 16S rRNA. All sequences were deposited in NCBI database (National Center for Biotechnology Information) (Table 2). Thirty-five species of mesophilic bacteria belonging to 14 genera were identified and comprised 17 species isolated from OA and MN, and 15 species from CV variety. The abundance of each micro-organism was calculated in relation to the total population in each stage of the processing. The lactic acid bacteria population was less diversified in terms of species. Six LAB species were identified, belonging to three genera. Four LAB distinct species were identified in OA, five were identified in MN, and three in the CV variety (Table 2).

All bacterial strains identified in the study were deposited in the Culture Collection of Agricultural Microbiology (http://www.ccma.dbi.ufla.br) and were coded as CCMA (as listed below). The species found in the all three varieties were *Bacillus cereus* group CCMA 1219-

1227, 1243, 1275-1276*; Cellulosimicrobium cellulans* CCMA 1165-1191*; Enterobacter cloacae* CCMA 1159-1164; *Lactobacillus plantarum* group CCMA 1058-1080*; Leuconostoc mesenteroides* CCMA 1081-1127 and *Enterococcus hirae* CCMA 1130, 1132-1140.

Some species were found only in one of the Ouro Amarelo varieties: *Acetobacter indonesiensis* CCMA 1129; *Arthrobacter luteolus* CCMA 1158; *Bacillus asahii* CCMA 1247; *B. clausii* CCMA 1229-1231, 1283*; B. slicheniformis* CCMA 1233; *B. safensis* CCMA 1232, *Paenibacillus cookii* CCMA 1267; *P.konsidensis* CCMA 1253, *Staphylococcus warneri* CCMA 1146 and *Enterococcus casseliflavus* CCMA 1141.

In Mundo Novo variety, *Bacillus humi* CCMA 1217; *B. simplex* CCMA 1216, *Brevibacillus parabrevis* CCMA 1218; *Gluconobacter oxydans* CCMA 1145; *Lysinibacillus macroides* CCMA 1280-1281*; Microbacterium testaceum* CCMA 1151; *Paenibacillus lactis* CCMA 1268; *Pantoea dispersa* CCMA 1278*,*1201-1207, *Enterococcus faecium* CCMA 1131 and *Enterococcus faecalis* CCMA 1128 were present.

In Catuaí Vermelho variety *Arthrobacter gandavensis* CCMA 1156; *A. koreensis* CCMA 1157; *Microbacterium paraoxydans* CCMA 1152; *Rhizobium pusense* CCMA 1200; *Rhodococcus pyridinivorans* CCMA 1213-1214 and *R. rhodochrous* CCMA 1282 were the identified ones.

Some species prevailed over the others, such as *Bacillus megaterium* CCMA 1245 in OA variety, *B. subtilis* group CCMA 1234*, Pantoea dispersa* CCMA 1201-1207, 1278 and *P. vagans* CCMA 1208-1210 in MN variety and *B. subtilis* group CCMA 1235-1242, 1244 in CV variety. *Lactobacillus plantarum group, Lac. mesenteroides* and *Enterobacter hirae* were present in all varieties and at all collection points (Table 2).

Acids and sugars analysis

In general, sugar did not show significant differences in the collected samples. Except for fructose in the CV variety (ranging from 7.55 mg g^{-1} in pulped coffee to 12.09 mg g^{-1} in demucilled coffee) (Table 3). Regarding coffee varieties, CV showed differences from the other varieties. At the beginning of fermentation, CV showed lower fructose concentration (7.55 mg g^{-1}). In dried coffee, CV showed high glucose (8.60 mg g^{-1}) and fructose (13.57 mg g^{-1}) concentration and lower sucrose concentration (33.53 mg g^{-1}). Sucrose predominated throughout the process, being the main sugar present in dried coffee, showing concentrations of 40.06, 46.53, and 33.53 mg g^{-1}, in OA, MN, and CV, respectively, followed by fructose 8.67, 8.52, and 13.67 mg g^{-1}.

The main acids involved during coffee fermentation (citric, malic, succinic, and acetic acid) were detected and quantified (Table 3) and lactic, butyric, propionic, oxalic and tartaric acids were not detected in any of the samples. Citric acid occurred at the highest concentration, increasing from the start of fermentation to dried coffee. The maximum concentration of citric acid was 6.86, 8.94 and 5.69 mg g^{-1} in OA, MN, and CV respectively, in dried coffee (DC). The same behavior was observed for malic acid, reaching the final concentration of 1.04, 1.24 e 0.91 mg g^{-1} in OA, MN, and CV varieties, respectively. Succinic acid, unlike citric acid, showed a decrease from fermentation to drying. Similar behavior was observed for acetic acid, which showed a final concentration of 0.83, 0.83 and 0.62 mg g^{-1} in OA, MN, and CV varieties, respectively.

Table 2. Population of bacteria present in the coffee cherry and during wet processing.

Species identified	Log CFU per g	Accession number	Ouro Amarelo				Mundo Novo				Catuaí Vermelho			
			CC	PC	DMC	DC	CC	PC	DMC	DC	CC	PC	DMC	DC
Mesophilic bacteria														
Total Population (log CFU per g)			4.10	3.95	4.60	2.48	4.69	4.38	2.60	2.78	4.84	3.36	4.84	2.70
Acetobacter indonesiensis	2.00	BAMW01000001												
Arthrobacter gandavensis	3.46	n.s.												
Arthrobacter koreensis	2.60	AY116496												
Arthrobacter luteolus	2.30	BCQM01000025												
Bacillus asahii	2.95	AB109209												
Bacillus cereus group	4.19	AE016877												
Bacillus clausii	3.26	X76440												
Bacillus hornickiae	2.30	FR749913												
Bacillus humi	3.18	AJ627210												
Bacillus licheniformis	2.00	AE017333												
Bacillus megaterium	2.70	JJMH01000057												
Bacillus pumilus group	4.26	ABRX01000007												
Bacillus safensis	2.90	ASJD01000027												
Bacillus simplex	4.00	BCVO01000086												
Bacillus subtilis group	4.87	ABQL01000001												
Brevibacillus parabrevis	2.00	D78463												
Cellulosimicrobium cellulans	4.61	CAOI01000359												
Enterobacter asburiae	3.67	BBED01000197												
Enterobacter cloacae	3.38	CP009854.1												
Gluconobacter oxydans	2.00	X73820												
Lysinibacillus fusiformis	3.28	AB271743												
Lysinibacillus macroides	2.30	LGCI01000008												
Microbacterium paraoxydans	2.48	BCRH01000180												
Microbacterium testaceum	2.00	X77445												
Paenibacillus cookii	2.00	AJ250317												
Paenibacillus konsidensis	4.00	EU081509												
Paenibacillus lactis	2.00	AY257868												
Pantoea bagglomerans	3.26	AJ233423												
Pantoea dispersa	4.46	DQ504305												
Pantoea vagans	4.43	EF688012												
Rhizobium pusense	3.80	jgi.1102370												

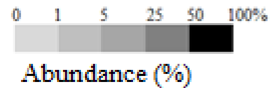
(continua…)

(conclusion of table 2).

Rhizobium radiobacter	4.01	AJ389904												
Rhodococcus pyridinivorans	3.30	LRRI01000001												
Rhodococcus rhodochrous	3.30	n.s.												
Staphyococcus warneri	2.30	L37603												

Species identified	**Log CFU per g**	**Accession number**	**Ouro Amarelo**				**Mundo Novo**				**Catuaí Vermelho**			
			CC	**PC**	**DMC**	**DC**	**CC**	**PC**	**DMC**	**DC**	**CC**	**PC**	**DMC**	**DC**
Acid lactic bacteria														
Total Population (log CFU per g)			6.15	5.54	4.07	3.11	5.49	4.66	3.84	3.75	3.63	3.96	4.98	3.96
Lactobacillus plantarum grupo	5.31	ACGZ01000098												
Leuconostoc mesenteroides	6.20	CP012009												
Enterococcus hirae	3.37	CP003504												
Enterococcus casseliflavus	2.00	n.s.												
Enterococcus faecium	2.00	AJKH01000109												
Enterococcus faecalis	3.00	ASDA01000001												

*Database accession number of EzTaxon (http://www.ezbiocloud.net/); n.s.: not sequenced, identified by MALDI-TOF with a score above 2.000; CC: coffee cherry; PC: pulped coffee; DMC: demucilled coffee; DC: dried coffee.



Volatile compounds

Gas chromatography, coupled with mass spectrometry (GC-MS), in combination with headspace solid phase micro-extraction (HS-SPME) detected 100 volatile compounds. Among these, 53 were detected in green coffee (Supplementary material) and 55 in roasted coffee (Supplementary material).

The 53 compounds identified in green coffee were aldehydes (16), alcohols (13), acids (5), hydrocarbons (5), ketones (4), esters (4) lactones (2), phenols, furans, pyrazines, and terpenes (1 each). A few novel volatile metabolites (1 in OA and 5 in MN) were detected after fermentation in the tank, compared to the unfermented beans. The subsequent drying step has a more significant impact on the volatile profile, yielding 12 (MN, CV) and 14 (OA) novel metabolites belonging to several different chemical classes. The compounds heptanoic acid and 2-ethyl-1-hexanol were detected only in beans of the variety OA. No qualitative differences were found in the volatile profiles of MN and CV in the dried beans.

Among the 55 compounds detected in roasted coffee were pyrazines (14), ketones (9), pyrroles (7), furans (4), furaldehydes (3), lactones (3), aldehydes, esters, hydrocarbons and phenols (2 each), acids, alcohols, pyrans, pyridines, terpenes, triazoles and thiophenes (1 each).

Sensory analysis

Two different sensory analyses were carried out to describe the flavor characteristics of the coffee beverages. The cupping test (Fig. 1) and Temporal Dominance of Sensations (TDS) were used to characterize some attributes of each coffee variety (Fig. 2).

Table 3. Carbohydrates and organic acids during the wet processing of Ouro Amarelo, Mundo Novo e Catuaí Vermelho varieties, effects of different samples and *p* value.

Compounds (mg g^{-1}) and Varieties	Samples			*p* value			
	Fermentation						
	Pulped coffee (PC)	Demucilled coffee (DMC)	Dried coffee (DC)	Variety (V)	Samples (S)	V × S	SEM*
Glucose							
Ouro Amarelo	7.69 aA	5.28 aB	4.31 bB				
Mundo Novo	7.90 aA	5.53 aB	3.63 bC	<0.01	<0.01	<0.01	0.27
Catuaí Vermelho	8.83 aA	4.30 aB	8.60 aA				
Fructose							
Ouro Amarelo	9.48 aB	11.69 aA	8.66 bB				
Mundo Novo	9.48 aB	12.19 aA	8.52 bB	0.06	<0.01	<0.01	0.31
Catuaí Vermelho	7.55 bB	12.09 aA	13.57 aA				
Sucrose							
Ouro Amarelo	11.63 aB	10.35 aB	40.06 aA				
Mundo Novo	11.76 aB	14.74 aB	46.54 aA	0.40	<0.01	0.01	1.44
Catuaí Vermelho	17.37 aB	21.03 aB	33.53 bA				
Citric acid							
Ouro Amarelo	3.85 aB	2.94 aB	6.86 bA				
Mundo Novo	3.66 aB	3.89 aB	8.94 bA	0.07	<0.01	<0.01	0.25
Catuaí Vermelho	4.64 aA	4.54 aA	5.69 aA				
Malic acid							
Ouro Amarelo	0.55 aB	0.44 aB	1.04 bA				
Mundo Novo	0.48 aB	0.35 aB	1.24 aA	0.95	<0.01	<0.01	0.02
Catuaí Vermelho	0.63 aB	0.52 aB	0.91 bA				
Succinic acid							
Ouro Amarelo	2.61 bA	0.74 bB	2.14 aA				
Mundo Novo	3.76 aA	3.85 aA	1.78 aB	<0.01	<0.01	<0.01	0.18
Catuaí Vermelho	4.42 aA	3.37 aB	1.66 aC				
Acetic acid							
Ouro Amarelo	1.55 bA	1.04 cA	0.83 aA				
Mundo Novo	2.23 aB	7.00 aA	0.83 aC	<0.01	<0.01	<0.01	0.11
Catuaí Vermelho	2.65 aA	2.10 bA	0.62 aB				

Data are presented as mean. a-b For each column, mean values with different low erase letters are significant at $p < 0.05$ by Scott–Knott test. A-B For each row, mean values with different capital letters are significant at $p < 0.05$ by Scott–Knott test.*Standard error of the means. PC: pulped coffee; DMC: demucilled coffee; DC: dried coffee.

The final score of the cupping test was 83.04, 81.50 and 82.33 for the OA, MN, and CV varieties, respectively. The variety OA showed higher scores for all attributes except for the balance, which was higher for the variety CV (Fig. 1). Panelists characterized the coffee from OA beans by the descriptors chocolaty, vanilla, creamy body, clean and pleasant finish. Mundo Novo was described as milk chocolate, almonds and pleasant finish. Catuaí Vermelho was defined as caramel flavor, citric acidity, creamy body and long finish.

The TDS curves (Fig. 2) showed the descriptions of the sensations as sweet, bitter chocolate, nuts, astringent, acidic, fruity and bitter, in each variety. Acidity was the dominant attribute in the OA variety (5 to 15 seconds). The dominant sensation in MN coffee was bitter at first (5 to 20 seconds), followed by bitter chocolate (20 to 25 seconds) and ending with nuts (29 seconds). The variety CV presented acidity as the dominant sensation at the beginning of the analysis (between 5 and 10 seconds) followed by astringency (between 10 and 15 seconds) and ending with a dominant sweet sensation (15 to 25 seconds). This sweetness may correspond with the caramel aroma observed by the tasters in the cupping test. However, a little astringency was observed in this variety, which was not desirable.

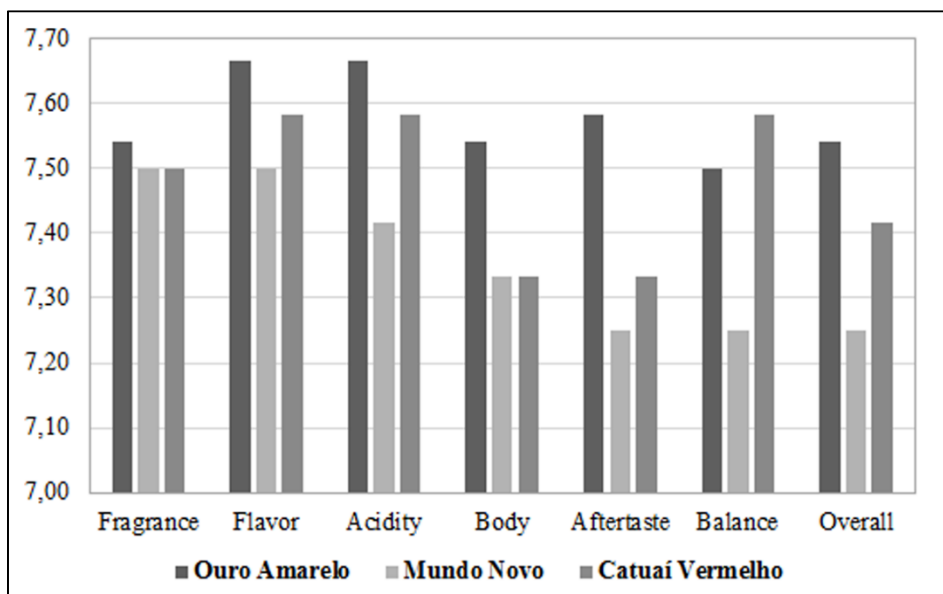


Figure 1. Scores of cup test (SCAA) of coffee processed by wet fermentation of varieties Ouro Amarelo, Novo Mundo and Catuaí Vermelho. Data are presented as mean. Standard deviation of mean ranged from 0.38 to <0.01.

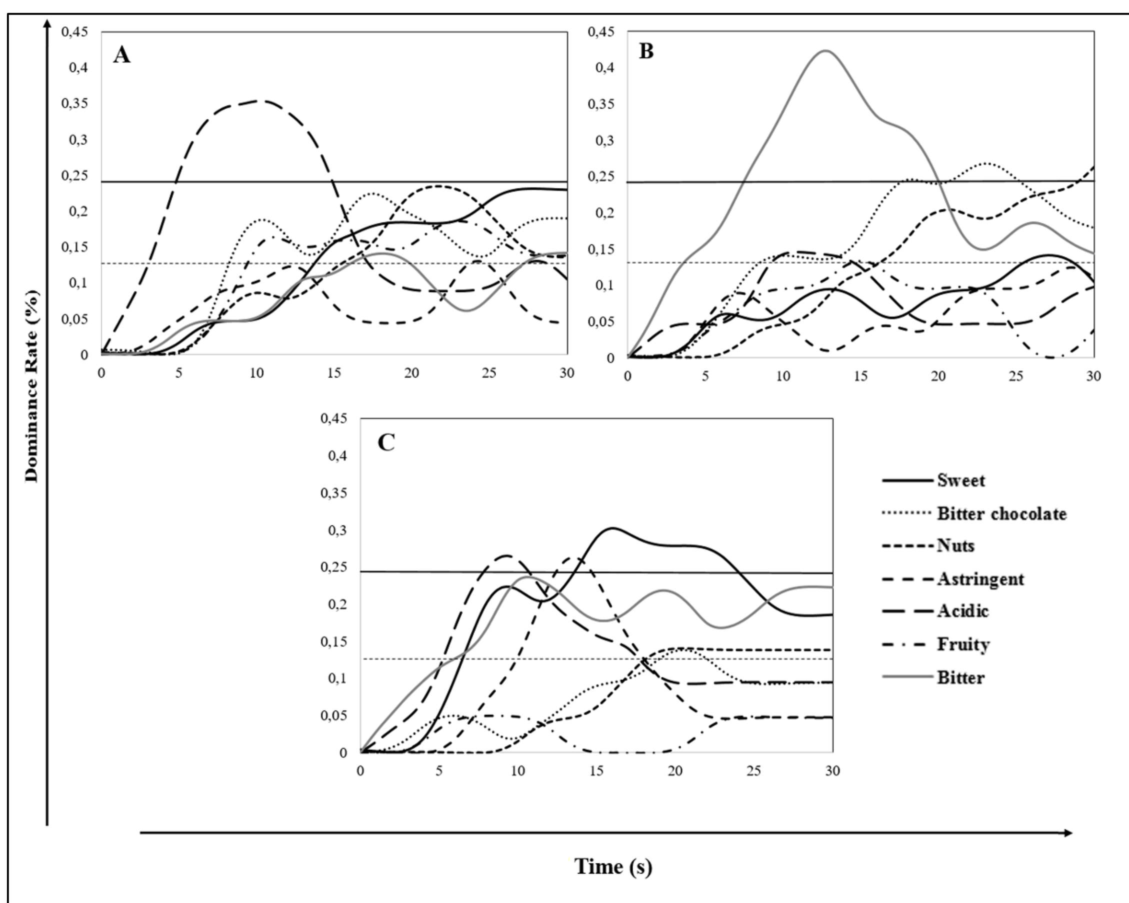


Figure 2. Temporal Dominance of Sensation (TDS) curves of the coffee processed by wet fermentation of Ouro Amarelo (A), Mundo Novo (B), and Catuaí Vermelho (C) varieties.

Discussion

One of the major issues involving the wet fermentation is the process control and determination of the ending point of fermentation. In the present study, the fermentation time was between 18 and 23 hours, which is generally recommended for wet processing. A decisive factor in achieving coffee quality in wet process is fermentation time; therefore, fermentations lasting more than 40 hours are not desirable (Quintero and Molina 2015). The ending point of fermentation can be determined based on either observations or empiric measurements. The measurement of pH value was established as a parameter to determine the fermentation ending. The lowering of pH value was due to microbial metabolism(Silva et al. 2008). Bacteria and yeasts present in coffee produced a considerable quantity of acids, specialty citric acid, as shown in Table 2 and 3.

Bacteria are the main microbiota group present during wet processing of coffee(Avallone et al. 2001). According to Avallone et al.(2002) and Hamdouche et al (2016), lactic acid bacteria had asignificant influence on the final coffee quality; besides, these bacteria might be used as microbialmarkers of wet fermentation. Here, LAB presented a larger population than mesophilic bacteria. LAB may perform alternative pathways of use of pyruvate under specific growth conditions. Compounds such as diacetyl, acetoin and consequently 2,3-butanediol, can be formed from the degradation of pyruvate; these produce fruity, creamy and buttery aromas (Von Wright and Axelsson 2012) In this work, 2,3 butanediol was detected in all varieties in the dried coffee and possibly, these compounds influenced the sensory results of our final product. Among the coffee varieties studied, the variety MN showed the highest diversity of LAB. *L. mesenteroides* was the main species found in wet process, reaching

values of 6 log CFU per g. This species was also reported by Evangelista et al. (2015) and Vaughan et al. (2015) in coffee samples.

MALDI-TOF MS was used for clustering of bacterial isolates. Some isolates could not be identified at the species level and representatives of each group and isolates with score >1.70 (accurate identification to the genus level) were selected for ribosomal region sequencing to confirm the species. MALDI-TOF MS is an effective method for the identification and detection of different microbial groups and has been applied in various areas(Santos et al. 2015; Pavlovic et al. 2012).

The highest diversity of the species identified for MN and CV varieties was observed in the coffee fruit, while in the OA variety, it was at the beginning of the fermentation (Table 2). The MN variety showed the highest concentration of carbohydrates (Table 3), which might justify the great variety of species found. The species present during the wet fermentation of coffee might vary according to the region where the fermentation is carried out (Evangelista et al. 2015) and, as noted here and for the first time reported according to coffee variety.

The main genera of mesophilic bacteria found were *Enterobacter, Cellulosimicrobium, Pantoea,* and *Bacillus*. These genera are usually found in wet fermentation, and among mesophilic bacteria(Evangelista et al. 2015; Lee et al. 2015; Vaughan et al. 2015). However, the present study isolated bacteria from the genus *Arthrobacter, Microbacterium* and *Paenibacillus,* which have already been described in the literature as commonly found in natural and semi-dry process (Evangelista et al. 2014a, b; Sakiyama et al. 2001; Silva 2015).

The temperature of the fermentation tank increased in proportion to the ambient temperature. The qualitative analysis of the volatiles after fermentation and dried coffee indicated that metabolic activity of microorganisms occurred during both steps of the

process in the present study. Changes in the production of compounds can be explained by different factors such as specific metabolites, it cannot be inferred whether the compound is a product of microbial or plant metabolism.

Fructose and glucose concentration decreased at the end of the fermentation process (Table 3), while sucrose increased throughout the process. Recently, Cheng et al. (2016), described the importance of these sugars in coffee processing, since these carbohydrates are essential precursors for volatile and non-volatile compounds, such as furans, pyrazine, aliphatic acids and hydroxymethyl furfural. It is still good to emphasize that glucose is an important precursor of citric acid(Papagianni et al. 1999) which is the main acid detected in the present study.

Acidity is often much desired in coffee and the highest score for acidity were detected in the OA variety, described as citric acid-like. This could be due to the presence of *Bacillus*, especially *B. licheniformis*, which are known producers of citric acid (Soccol et al. 2006). The nuts sensation perceived might correspond to the almond aroma that was recognized in the same beverage by the tasters in the cupping test.

It seems that coffee varieties might be a greater influence than the environmental conditions, for bacteria diversity and volatile compounds. The composition of the volatiles in the roasted bean may be improved using starter cultures (Evangelista et al. 2014a, b; Lee et al. 2015). We suggest that isolates identified in the study could be tested as starter cultures from these varieties.

Secondary metabolites produced during fermentation and drying could also directly or indirectly influence coffee aroma (Lee et al. 2015). All treatments were considered as specialty coffees, as the SCA scores were above 80, which is consistent with the compounds identified by GC-MS analysis (Supplementary material), where compounds were found to impart flavor to the final product. The TDS analysis showed

that despite having the same scores, samples from different varieties caused different sensorial sensations, which may be the influence of microbiota present and metabolites produced (Table 3).

Conclusion

The dominant and common bacteria found in wet fermentation in this work were *Enterobacter cloacae*, *Leuconostoc mesenteroides* and *Lactobacillus plantarum*. *L. mesenteroides* showed high dominance over the other species, and probably have an important influence in wet coffee fermentation. The bacteria diversity during the fermentation of the three coffee varieties (Ouro Amarelo, Mundo Novo and Catuaí Vermelho) provided useful information about the possible starter cultures that can be studied in wet processing of coffee.

The knowledge of the chemical composition of green and roasted coffee also helped the selection of a more suitable coffee variety for wet fermentation. The sensorial analysis of the coffee using two techniques (cup test and TDS) allowed a better description of the sensorial characteristics of the varieties. These differences may be linked to the microbiological quality of each variety.

Conflict of Interest

We state that there are no conflict of interest exists in the submission of this manuscript, and manuscript is approved by all authors for publication.

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ARTIGO 3: Mesophilic and lactic acid bacteria as starter culture in Arabica coffee fermentation

ABSTRACT

The inoculation of starter cultures aims to standardize the fermentation process and obtain products with higher quality and safety. The objectives of this work were to select and evaluate the fermentative performance of mesophilic and lactic acid bacteria in pulped coffee. One hundred and four isolates (77 mesophilic bacteria - MB and 27 lactic acid bacteria - LAB) were evaluated. Bacteria were selected for the production capacity of pectinase enzymes, production of organic acids and growth in a medium made with coffee and pH lowering. Nine strains of bacteria (five mesophilic and four acid lactic bacteria) were selected and used as starter culture in 3 kg of pulped coffee. The population count of mesophilic and lactic acid bacteria, concentration of organic acids and presence of volatile compounds was evaluated. *Pantoea dispersa* CCMA 1203; *Cellulosimicrobium cellulans* CCMA 1186, *Leuconostoc mesenteroides* CCMA 1105 and *Lactobacillus plantarum* CCMA 1065 presented some volatile compounds thought fermentation that can improve the quality of beverage, such as isovaleric acid, 2, 3 –butanediol; phenethyl alcohol; β -linalool; ethyl linoleate; and ethyl 2-hydroxypropanoate. Future work should also be conducted to evaluate these strains in different coffee varieties and coffee producing areas.

1 Introduction

The natural fermentation of coffee is a simple process of degradation of the mucilage layer, which is rich in polysaccharides (pectin) and less complex substances for formation of taste and flavor precursors (Batista et al., 2016). Coffee fruits contain endogenous enzymes that degrade the mucilage layer, but this activity is not sufficient for a complete and adequate mucilage removal process (Agate and Bhat, 1966). The mucilage is degraded by fermentation that allows the growth of microorganisms

producing enzymes. Some enzymes such as polygalacturonase (PG) that catalyzes the hydrolysis of 1,4-glycosidic bonds in polygalacturonic acid; pectin lyase (PL) which acts by catalyzing pectin breakdown by releasing galacturonic unsaturated acids. The third enzyme is pectin methyl esterase (PME) responsible for de-esterification of the methoxy group of pectin forming pectic acid and methanol (Agate and Bhat, 1966; Massawe and Lifa, 2010).

The volatile profiles and subsequently, the aroma profiles and cupping qualities of roasted coffee are very much dependent on the composition of aroma precursors present in green coffee beans prior to roasting. The extracellular enzymes and organic acids produced from yeasts and lactic acid bacteria, respectively, could potentially lead to the hydrolysis of macromolecules such as carbohydrates, proteins and polyphenols, generating important aroma precursors such as reducing sugars, amino acids and chlorogenic acids. Since aroma precursors in green coffee beans play an essential role in the formation of volatile compounds associated with coffee aroma during roasting, differences in the concentrations of aroma precursors will indirectly correspond to differences in the volatile and aroma profiles of roasted coffee. This interdependence between the non-volatile constituents of the green coffee beans and the corresponding aromatic profile of the roasted coffee feeds the idea that changes in the non-volatile composition of the green coffee beans caused by fermentative processes may have a possible impact on the coffee flavor (Lee et al., 2015).

Microorganisms have already been used as starter cultures in fermentative processes for thousands of years. The evolution of taxonomy and identification methods has led to a varied extension of the use of these microorganisms in foods (Bourdichon et al., 2012). The use of yeasts as starter cultures in coffee has been reported by researchers (Evangelista et al., 2014; Ribeiro et al., 2017; Gelvez et al., 2017), however,

the use of bacteria is still restricted. There are numerous possibilities of bacterial species that can and should be exploited as starter cultures.

Using coffee starter cultures, it is possible to cover new opportunities to produce coffee with different aromas and flavors, leading to new perspectives on coffee quality. The relationship between coffee fermentation and aroma profile is very close (Gonzalez-Rios et al., 2007; Jackels et al., 2006; Jackels and Jackels, 2005; Lin, 2010). With optimized parameters and starter cultures appropriate for the removal of mucilage, fermentation may confer desirable attributes to coffee. The targeted use of different species of bacteria would lead to a greater diversification of flavors, which is already evident in the beer and wine industry (Marshall and Mejia, 2011). Therefore, the objective of this work was to select bacteria (mesophilic and lactic acid group) strains isolated from coffee capable of producing organic acids and volatile compounds aiding the fermentation through the wet process of coffee.

2 Material and methods

2.1 *Microorganisms and fermentative performance in culture medium with coffee*

Bacteria isolates from processed coffee were obtained from Culture Collection of Agricultural Microbiology (CCMA, Federal University of Lavras, Lavras, Minas Gerais, Brazil). The microorganisms have been previously isolated and identified from coffee fruit (*Coffea arabica* L. var. Ouro Amarelo, Mundo Novo and Catuaí Vermelho) during wet fermentative processes. A total of 104 isolates (including 77 MB and 27 LAB) were evaluated potential for starter cultures (Table 1). Mesophilic bacteria strains were maintained on nutrient broth (Himedia, Mumbai, India) and lactic acid bacteria were maintained on MRS broth (Merck, Darmstadt, Germany) stored in an ultra-freezer at -80 °C, containing 20% glycerol (w/w).

Table 1. Bacterial isolates used in fermentative performance in culture medium containing coffee peel and pulp.

Nº	Strain	Species	Nº	Strain	Species
Mesophilic Bacteria (MB)					
1	CCMA 1234	*Bacillus subtilis group*	41	CCMA 1269	*Paenibacillus illinoisensis*
2	CCMA 1244		42	CCMA 1253	*Paenibacillus konsidensis*
3	CCMA 1236		43	CCMA 1267	*Paenibacillus cookii*
4	CCMA 1237		44	CCMA 1268	*Paenibacillus lactis*
5	CCMA 1240		45	CCMA 1153	*Microbacterium paraoxydans*
6	CCMA 1238		46	CCMA 1152	
7	CCMA 1239		47	CCMA 1212	*Rhizobium radiobacter*
8	CCMA 1241		48	CCMA 1200	*Rhizobium pusense*
9	CCMA 1242		49	CCMA 1182	
10	CCMA 1235		50	CCMA 1183	*Cellulosimicrobium cellulans*
11	CCMA 1228		51	CCMA 1173	
12	CCMA 1232	*Bacillus safensis*	52	CCMA 1184	
13	CCMA 1250	*Bacillus pumilus*	53	CCMA 1175	
14	CCMA 1272		54	CCMA 1187	
15	CCMA 1274		55	CCMA 1188	
16	CCMA 1248		56	CCMA 1189	
17	CCMA 1251		57	CCMA 1179	
18	CCMA 1249		58	CCMA 1180	
19	CCMA 1273		59	CCMA 1190	
20	CCMA 1252		60	CCMA 1185	
21	CCMA 1245	*Bacillus megaterium*	61	CCMA 1186	
22	CCMA 1246				
23	CCMA 1229	*Bacillus clausii*	62	CCMA 1203	*Pantoea dispersa*
24	CCMA 1230		63	CCMA 1278	
25	CCMA 1283		64	CCMA 1207	
26	CCMA 1231		65	CCMA 1277	*Pantoea agglomerans*
27	CCMA 1233	*Bacillus licheniformis*	66	CCMA 1211	
28	CCMA 1247	*Bacillus asahii*	67	CCMA 1208	*Pantoea vagans*
29	CCMA 1265	*Bacillus horneckiae*	68	CCMA 1209	
30	CCMA 1266		69	CCMA 1210	
31	CCMA 1264	*Lysinibibacillus fusiformis*	70	CCMA 1150	*Microbacterium testaceum*
32	CCMA 1263		71	CCMA 1147	
33	CCMA 1280	*Lysinibacillus macroides*	72	CCMA 1148	
34	CCMA 1281		73	CCMA 1151	
35	CCMA 1218	*Brevibacillus parabrevis*	74	CCMA 1213	*Rhodococcus pyridinivorans*
36	CCMA 1215	*Psychrobacillus psychrotolerans*	75	CCMA 1214	
37	CCMA 1157	*Arthrobacter koreensis*	76	CCMA 1282	
38	CCMA 1279				
39	CCMA 1217	*Bacillus humi*			
40	CCMA 1216	*Bacillus simplex*			

(continue…)

(conclusion of table 1)

Lactic Acid Bacteria (LAB)					
Nº	**Strain**	**Species**	**Nº**	**Strain**	**Species**
1	CCMA 1082	*Leuconostoc mesenteroides*	15	CCMA 1117	*Leuconostoc mesenteroides*
2	CCMA 1083		16	CCMA 1120	
3	CCMA 1084		17	CCMA 1110	
4	CCMA 1085		18	CCMA 1058	*Lactobacillus plantarum*
5	CCMA 1087		19	CCMA 1059	
6	CCMA 1089		20	CCMA 1064	
7	CCMA 1090		21	CCMA 1065	
8	CCMA 1105		22	CCMA 1067	
9	CCMA 1106		23	CCMA 1071	
10	CCMA 1108		24	CCMA 1072	
11	CCMA 1109		25	CCMA 1076	
12	CCMA 1111		26	CCMA 1078	
13	CCMA 1112		27	CCMA 1080	
14	CCMA 1115				

The fermentations with bacteria were carried out in culture medium with coffee as described in Silva et al. (2013). For preparation of medium (40 g of coffee cherries was homogenized in Stomacher with 1 L of distilled water for 10 min. The medium was filtered and added 0.5% of glucose, and boiled for 10 min. The pH of the medium was adjusted to 5.5 with HCl solution).

The strains tested were grown in liquid broth nutrient medium and MRS until reaching the population of 10^7 CFU/ml. Cells were separated by centrifugation (9000 rpm, 4 °C, 10 min) and washed twice with 0.1% sterile peptone water. The inoculum was transferred to the fermentation medium (100 mL) and the vials were kept at room temperature (approximately 30 °C) for 48 hours. Samples were collected every 12 hours to evaluate the fermentation process. The pH was determined by potentiometric. Bacteria population, organic acid concentration and enzymatic activity were evaluated. The fermentations were carried out in duplicate.

2.2 Controlled fermentation

For controlled fermentation coffee cherries of the variety Catuaí Vermelho were collected mechanically on a farm located in Lavras, Minas Gerais State, Brazil, at 900 meters above sea level. Three kilograms of pulped coffee was fermented in open buckets with 2 L of sterile water. The strains chosen were inoculated at population of 10^7 CFU per gram of coffee. The fermentation was performed in duplicate. The total fermentation time was 48 hours (BATISTA et al., 2016). After fermentation, the water was discarded, and coffee was transferred to suspended terraces for sun drying until it reached 11–12% of moisture content (w.b.). Samples were collected in initial of fermentation, 12 hours (T12), 24 hours (T24) and 48 hours (T48) of fermentation and dried coffee for chemical and microbiology analysis.

2.3 Mesophilic and lactic acid bacteria count

Bacterial population of coffee culture medium was quantified by spread-plate method. Serial decimal dilutions were performed with sterile peptone water (0.1% bacteriological peptone [Himedia, Mumbai, India]). Mesophilic bacteria were quantified by plating on Nutrient Agar (Himedia, Mumbai, India) and LAB on MRS agar (Merck, Darmstadt, Germany). Plates were incubated at 30ºC and 35°C for 48 h.

2.4 Pectinase activity

The pectinolytic activity of the 104 strains was determined as described by Schwan et al. (1997), Hanckin and Lacy (1984) and Silva et al. (2013). The strains were grown on plates containing MP5 mineral medium (0.5% glucose; 0.5% polygalacturonic acid; 0.6% KH2PO4; 0.1% yeast extract; 0.2 (NH4) SO; 1.5% agar and 0.1 mL of the solutions, FeSO4 0.0001%; MgSO4 0.02%; (0.0002% CaCl2,

0.0002% H3BO3, MnSO4 0.0002%, ZnSO47H2O 0.0014%, CuSO45H2O 0.001%, MoO3 0.0002%) for determination of the activity of the polygalacturonase (PG) enzyme. The determination of the activity of the pectin lyase (PL) enzyme was carried out using MP7 medium having the same constitution as described above with substitution of polygalacturonic acid for citrus pectin.

The enzymatic assay was indicated as positive by the formation of clear halo around the colonies after precipitation of polygalacturonic acid and pectin with 1% cetyl triethyl ammonium bromide (CTAB) (Sigma-Aldrich [Germany]) solution. The yeast *Kluyveromyces marxianus* CCT 3172 (Schwan et al., 1997) was used as the positive control.

2.5 ***Quantification of pectin lyase (PL) and pectin methyl esterase (PME) activity***

The activity of pectin lyase and pectin methyl esterase were quantified in culture medium with coffee only of strains that showed positive results for qualitative tests. The pectin lyase (PL) activity was determined by increasing the absorbance (Albersheim, 1996), using the extinction coefficient of 5550 $M^{-1} cm^{-1}$. A pectin solution (3 mL citrus pectin 2.5% in 100 mM phosphate buffer, pH 6.8) and 4.5 mL of culture supernatant. The mixture was incubated at 40 °C and 0.5 mL was collected at 0, 10, 15, 20 and 30 minutes, and each time the reaction was quenched by addition of 4.5 mL of 0.01M HCl. The samples were analyzed in spectrophotometer at 235 nm. The equipment been reset with pectin solution and culture medium with coffee without inoculation.

The PL activity was expressed from the calculation of the galacturonides concentration released by the PL action:

$A = \varepsilon \times l \times C$,

where A, the absorbance of the sample at 235 nm; ε, the molar extinction coefficient (M^{-1} cm^{-1}); 1 (one), the optical path (1 cm); and C, analyte concentration, expressed in M.

Pectin methyl esterase activity (PME) quantification was determined by titration according to the methodology proposed by Baracat et al. (1989). The reaction was carried out with 3 mL of the culture supernatant mixed with 20 mL of citric pectin 1% (60% methoxylated or higher) in NaCl 0.1M solution. The pH 7.5 was adjusted with NaOH 0.5M. This solution was and incubated for 30 minutes at 30 ºC and the pH maintained at 7.5 by addition of NaOH 0.02M. The PME activity was proportional to the NaOH volume spent to keep the pH at 7.5. PME activity was expressed as the micro equivalents of polygalacturonic acid produced mL^{-1} h^{-1}.

2.6 Chemical analysis

2.6.1 ***Organic acids Determination***

Organic acids (malic, lactic, acetic, butyric, propionic, citric, oxalic, succinic and tartaric acid) were analyzed as described in Ribeiro et al. (2017).

The samples of fermented culture medium with coffee were centrifuged (10.000 rpm at 4 C for 10 minutes) and the supernatant was filtered using a 0.22 µm cellulose acetate filter. The extracts were analyzed using a high-performance liquid chromatography (HPLC) system (Shimadzu, Japan). A Shimpack SCR-101H (7.9 mm x 30 cm) column was used with a 100 mM solution of perchloric acid with flow rate of 0.6 ml/min as the mobile phase. The oven temperature was kept at 50 °C, detected with a 210 nm UV detector. The compounds quantification was performed using calibration curves with different standard concentrations analyzed using the same conditions as for the samples. Analyses were performed in duplicate.

2.6.2 Volatiles compounds

Analysis of volatile compounds were made in green and roasted coffee by gas chromatography/mass spectrometry (GC/MS). Volatile compounds were extracted using a manual headspace-solid phase microextraction procedure (HS–SPME) according to Evangelista et al. (2014b). The compounds were analyzed using a Shimadzu QP2010 GC model equipped with mass spectrometry (MS) and a silica capillary Carbo-Wax 20M (30 m × 0.25 mm x 0.25 mm) column. Operating conditions were performed as described by Ribeiro et al. (2017). 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 the retention index (RI) for each compound and compared with RI values found in the literature data (Czerny and Grosch, 2000; Eom and Jung, 2013; Piccino et al., 2014).

2.7 ***Statistical analysis***

The data organic acids were evaluated by variance analysis (ANOVA) and the Scott-Knott test was used for comparison between means. A 10 × 4 factorial arrangement of treatment was used to analyze the organic acids results: ten treatments (Control, CCMA 1234, CCMA 1251, CCMA 1186, CCMA 1203, CCMA 1151, CCMA 1082, CCMA 1105, CCMA 1065, CCMA 1072) and five times of collected samples (T0, T12, T24, T48, dried coffee). Data were analyzed under the following model:

$$X = \mu + Treat_j + Time_k + Var \times Samp_{jk} + e_{jk}$$

were, μ = global mean; $Treat_j$= treatment effect (j = Control, CCMA 1234, CCMA 1251, CCMA 1186, CCMA 1203, CCMA 1151, CCMA 1082, CCMA 1105, CCMA 1065, CCMA 1072); $Time_k$ = time collected effect (k = T12, T24, T48, dried coffee);

Treat × Time*jk* = effect of interaction between treatment and time collection time; e*jk* = experimental error. The tests were performed using Sisvar 5.6 software (Ferreira, 2014). Significance level was defined at $p<0.05$ level. The production of acids in medium culture made with coffee was obtained and analyzed by principal component analysis (PCA) using SensoMaker software (Nunes and Pinheiro, 2012), to evaluate differences and similarities among the samples and their characteristics.

3 Results and Discussion

3.1 *Evaluation of the fermentative performance of bacteria*

The criteria for bacteria selection with potential for starter culture in wet coffee were pH lowering, bacteria growth, pectinases production, organic acids (lactic, malic, succinic, citric and propionic acids) in culture medium containing coffee pulp.

The bacteria population and pH of culture medium containing coffee pulp are presented in Figures 1 and 2. Mesophilic bacteria lowered the pH values to around 4.5 (Fig 1). Lactic acid bacteria had the ability to lower the pH value reaching at the end around 3.5 (Fig 2). Lactic bacteria reduced the pH between 27 and 38%, while the mesophilic bacteria reduced by up to 36% in relation to initial value. The first criterion for selection of the starter bacteria was the ability to lower the pH of the coffee medium, and strains chosen for controlled fermentation reduced up to 32% of the pH in pulp and coffee peel medium. During coffee fermentation the pH goes down due to production of acids that are released in medium by microorganisms. Ability to survive in fermentation medium is an important feature for maintaining the starter culture.

Initial population inoculated was 7 log CFU/g for the two bacterial group. The MB population increased up to 37% compared to the initial population, while, the LAB population increased by up to 26%. In this work some strains showed reduction of their population, therefore were not chosen for controlled fermentation. This can be

explained due to growing conditions not being adequate to develop or maintain the population.

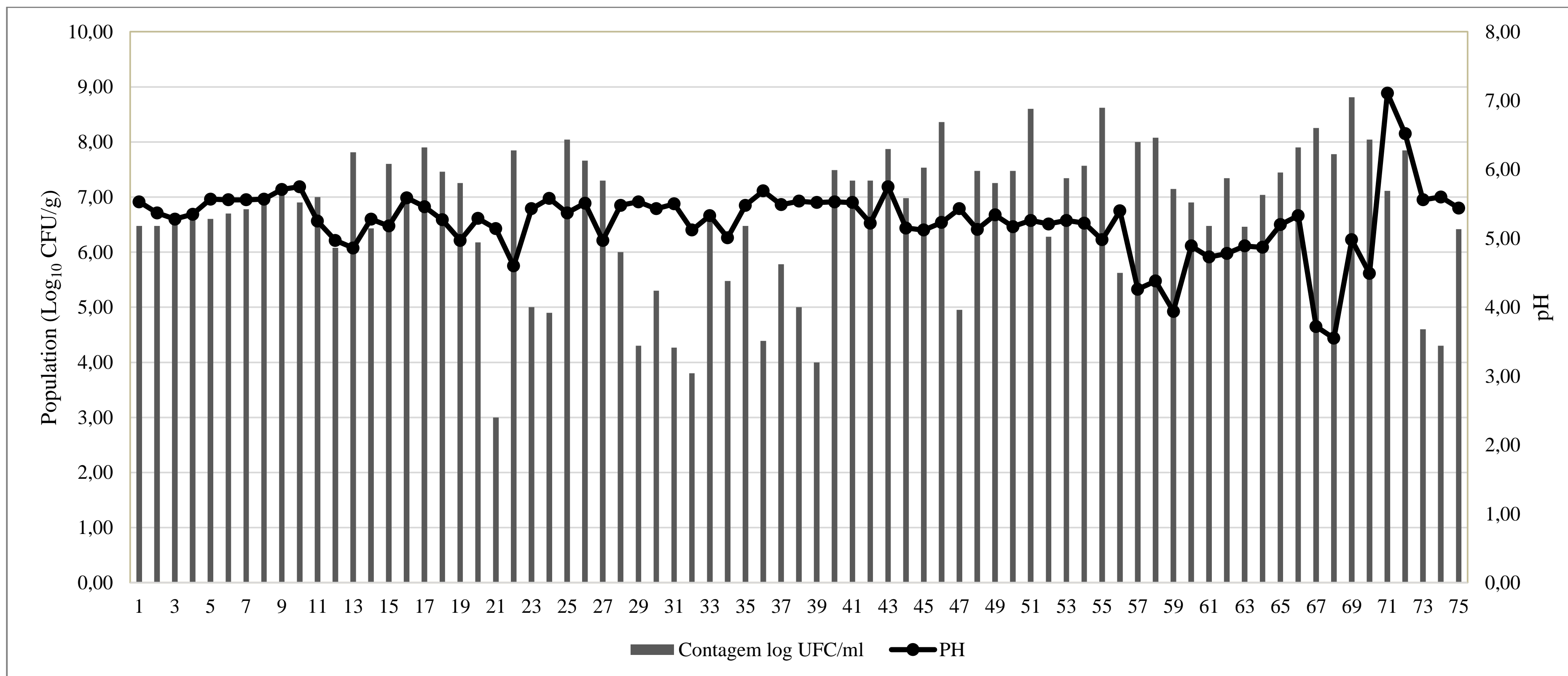

Figure 1. Mesophilc bacterial population (Log_{10} CFU/g) and pH of culture medium containing coffee pulp at final of fermentation. (1) CCMA 1234; (2) CCMA 1244; (3) CCMA 1236; (4) CCMA 1237; (5) CCMA 1240; (6) CCMA 1238; (7) CCMA 1239; (8) CCMA 1241; (9) CCMA 1242; (10) CCMA 1235; (11) CCMA 1228; (12) CCMA 1232; (13) CCMA 1250; (14) CCMA 1272; (15) CCMA 1274; (16) CCMA 1248; (17) CCMA 1251; (18) CCMA 1249; (19) CCMA 1273; (20) CCMA 1245; (21) CCMA 1246; (22) CCMA 1229; (23) CCMA 1230; (24) CCMA1283; (25) CCMA 1231; (26) CCMA 1233; (27) CCMA 1247; (28) CCMA 1265; (29) CCMA 1266; (30) CCMA 1217; (31) CCMA 1216; (32) CCMA 1269; (33) CCMA 1253; (34) CCMA 1267; (35) CCMA 1268; (36) CCMA 1264; (37) CCMA 1280; (38) CCMA 1281; (39) CCMA 1263; (40) CCMA 1218; (41) CCMA 1215; (42) CCMA 1182; (43) CCMA 1183; (44) CCMA 1173; (45) CCMA 1184; (46) CCMA 1175; (47) CCMA 1187; (48) CCMA 1188; (49) CCMA 1189; (50) CCMA 1179; (51) CCMA 1180; (52) CCMA 1190; (53) CCMA 1185; (54) CCMA 1186; (55) CCMA 1157; (56) CCMA 1279;

(57) CCMA 1203; (58) CCMA 1278; (59) CCMA 1207; (60) CCMA 1277; (61) CCMA 1211; (62) CCMA 1208; (63) CCMA 1209; (64) CCMA 1210; (65) CCMA 1150; (66) CCMA 1153; (67) CCMA 1147; (68) CCMA 1148; (69) CCMA 1151; (70) CCMA 1152; (71) CCMA 1212; (72) CCMA 1200; (73) CCMA 1213; (74) CCMA 1214; (75) CCMA 1282.

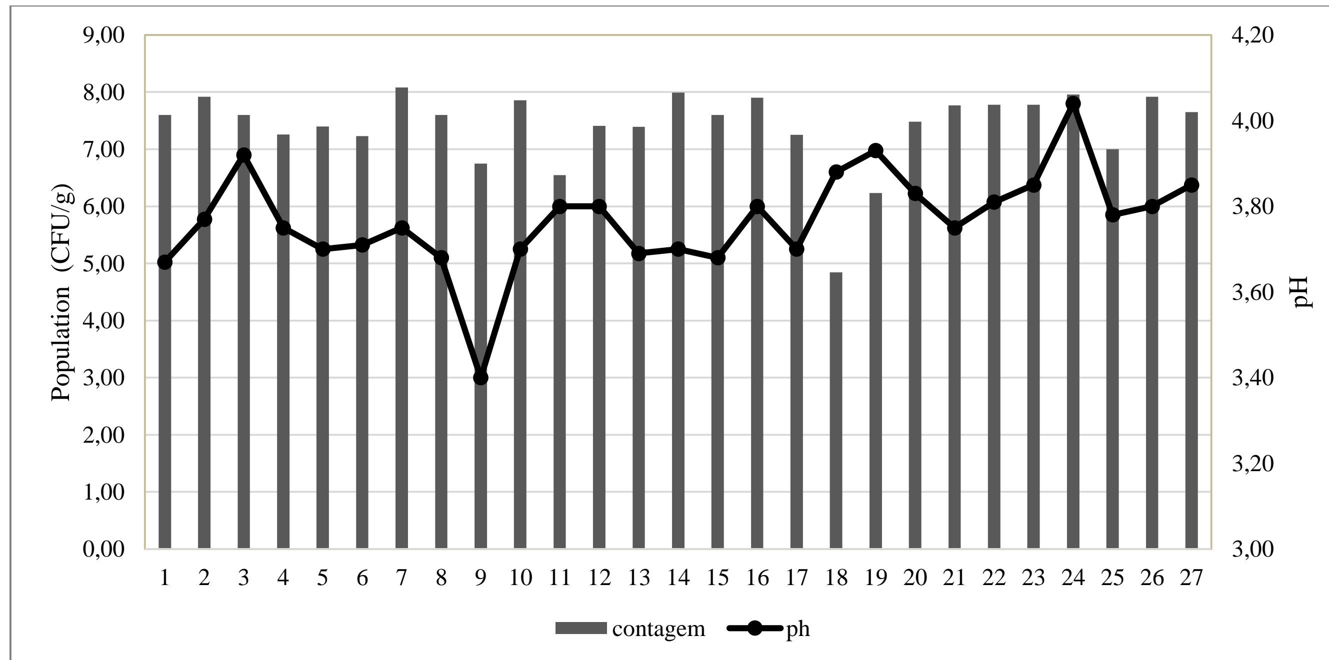

Figure 2. Lactic acid bacterial population (Log_{10} CFU/g) and pH of culture medium containing coffee pulp at final of fermentation. (1) CCMA 1082; (2) CCMA 1083; (3) CCMA 1084; (4) CCMA 1085; (5) CCMA 1087; (6) CCMA 1089; (7) CCMA 1090; (8) CCMA 1105; (9) CCMA 1106; (10) CCMA 1108; (11) CCMA 1109; (12) CCMA 1111; (13) CCMA 1112; (14) CCMA 1115; (15) CCMA 1117; (16) CCMA 1120; (17) CCMA 1110; (18) CCMA 1058; (19) CCMA 1059; (20) CCMA 1064; (21) CCMA 1065; (22) CCMA 1067; (23) CCMA 1071; (24) CCMA 1072; (25) CCMA 1076; (26) CCMA 1078; (27) CCMA 1080.

Of a total of 104 bacterial isolates that were evaluate, 22 strains showed a positive result for pectin lyase (PL) and four showed a positive result for polygalacturonase (PG). The quantification of pectin lyase (PL) and pectin methyl esterase (PME) activity in coffee medium were done with 17 isolates, whose halo was greater than 2 mm. Only mesophilic bacteria showed positive results for pectinolytic activity, and lactic acid bacteria were not producing pectinolytic enzymes. The species tested here produced enzyme activity halos with small diameter when compared to other study in which the production of pectinase enzymes of yeasts was tested, there strains capable of producing halos up to 30 mm were used (Silva et al. 2013).

Bacillus subtilis CCMA 1242 showed results for PL activity of 0.029 U/mL and PME activity of 0.383 microequivalent/$mL^{-1}h^{-1}$. *Bacillus subtilis* CCMA 1234 and *Paenibacillus konsidensis* CCMA 1242 were the major producers of pectin lyase (PL) (0.073 U/mL) (Table 3). *Pantoea dispersa* CCMA 1278, *Bacillus subtilis* CCMA 1238 and CCMA 1239 were the major pectin methyl esterase producer (PME) (0.633; 0.483 and 0.450 microequivalent /$mL^{-1}h^{-1}$, respectively).

The low pectinases production may have occurred due to i) the bacteria strains tested in this study were isolated from pulped and fermented coffee by wet processing. these strains were isolated from pulped coffee, therefore, there was not a large amount of pectic substrate to be degraded. There may have been a natural selection of strains that did not produce pectinases; ii) Some authors reported that pectinase enzymes can be suppressed by excess or lack of carbohydrates like some monosaccharides (Schwan et al., 1997; Masoud and Jespersen, 2006; Henick-Kling et al., 1998). iii) A third factor that may have influenced the lower enzymatic activity in culture medium with coffee may have been lacking in nitrogen source (Silva et al., 2013); iv) the last factor that could have interfered in the enzymatic production was rapid lowering of pH (Fig 1 and

Fig 2) due to the production of organic acids to be not conducive to enzymatic activity under acid conditions (pH 3.5-5.5) (Silva, 2015).

Table 2. Pectin lyase (PL) and pectin methyl esterase (PME) activity quantified in CPM medium fermented with mesophilic and lactic acid bacteria.

Nº	Strain	Species	PL[a]	PME[b]
1	CCMA 1234	*Bacillus subtilis*	0.073± 0.01c	0.100±0.01a
2	CCMA 1228		0.013±0.03a	0.250± 0.00b
3	CCMA 1236		0.064± 0.00c	0.070±0.00a
4	CCMA 1237		0.057± 0.02c	0.100±0.06a
5	CCMA 1238		0.033± 0.01b	0.483±0.03d
6	CCMA 1239		0.021± 0.00b	0.450±0.00d
7	CCMA 1240		0.024± 0.03b	nd
8	CCMA 1241		0.005±0.02a	0.250±0.00b
9	CCMA 1242		0.029±0.00b	0.383±0.03c
10	CCMA 1244		0.001±0.02a	0.250±0.00b
11	CCMA 1248	*Bacillus pumilus*	0.016±0.00a	nd
12	CCMA 1251		0.025±0.01b	nd
13	CCMA 1273		0.010±0.02a	0.250±0.00b
14	CCMA 1253	*Paenibacillus konsidensis*	0.073±0.01c	0.100±0.06a
15	CCMA 1211	*Pantoea agglomerans*	0.033±0.01b	0.070±0.04a
16	CCMA 1278	*Pantoea dispersa*	0.055±0.05b	0.633±0.03e
17	CCMA 1279	*Arthrobacter korensis*	0.001±0.00a	nd

[a](expressed as the amount of µmol of unsaturated galacturonides/min per ml of the culture supernatant U/mL); [b](expressed as microequivalents of pectic acid released per $ml^{-1}h^{-1}$).nd: not detected. a-emean values with different letters are significant at $p < 0.05$ for each treatment by Scott–Knott test.

Another important feature in selecting starter cultures for coffee fermentation is the production of metabolites (Figure 3).

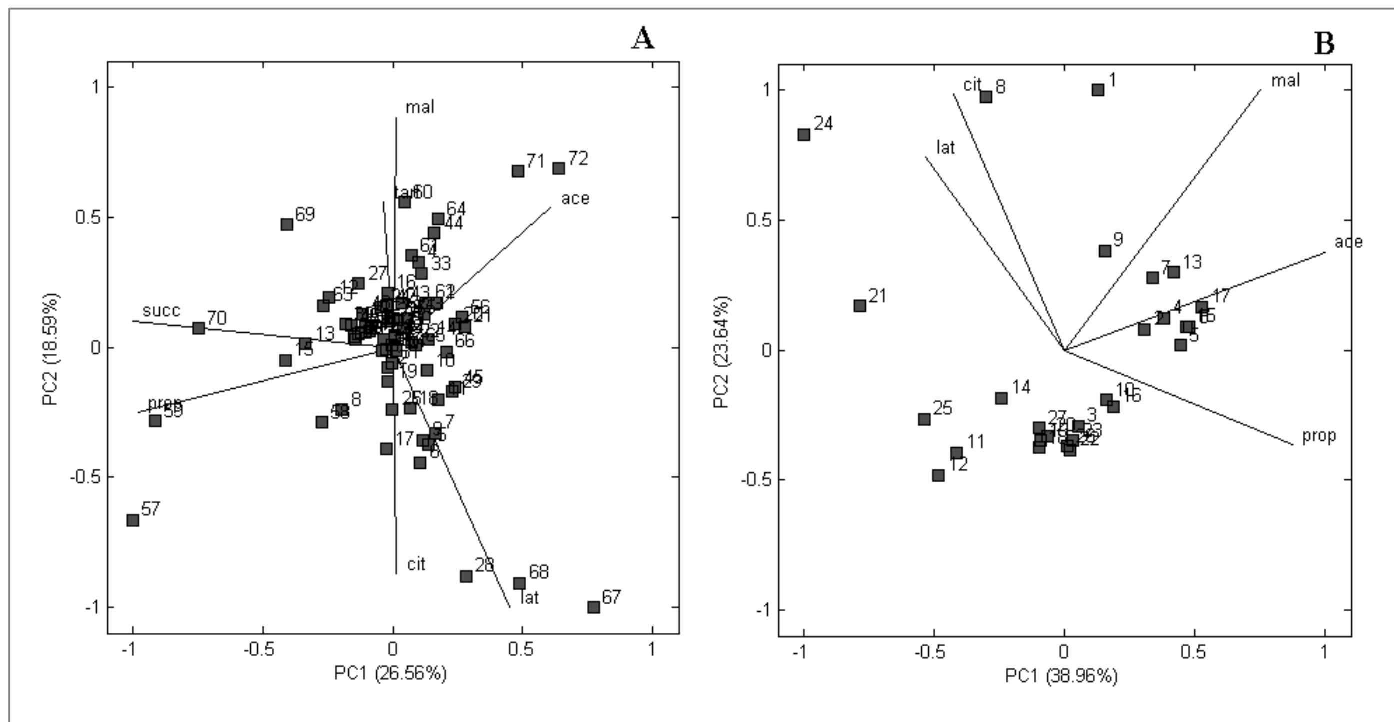

Figure 3. Principal component analysis loading plot for acid production in culture medium containing coffee pulp. **Inoculated with mesophilic bacteria (A):** (1) CCMA 1234; (2) CCMA 1244; (3) CCMA 1236; (4) CCMA 1237; (5) CCMA 1240; (6) CCMA 1238; (7) CCMA 1239; (8) CCMA 1241; (9) CCMA 1242; (10) CCMA 1235; (11) CCMA 1228; (12) CCMA 1232; (13) CCMA 1250; (14) CCMA 1272; (15) CCMA 1274; (16) CCMA 1248; (17) CCMA 1251; (18) CCMA 1249; (19) CCMA 1273; (20) CCMA 1245; (21) CCMA 1246; (22) CCMA 1229; (23) CCMA 1230; (24) CCMA1283; (25) CCMA 1231; (26) CCMA 1233; (27) CCMA 1247; (28) CCMA 1265; (29) CCMA 1266; (30) CCMA 1217; (31) CCMA 1216; (32) CCMA 1269; (33) CCMA 1253; (34) CCMA 1267; (35) CCMA 1268; (36) CCMA 1264; (37) CCMA 1280; (38) CCMA 1281; (39) CCMA 1263; (40) CCMA 1218; (41) CCMA 1215; (42) CCMA 1182; (43) CCMA 1183; (44) CCMA 1173; (45) CCMA 1184; (46) CCMA 1175; (47) CCMA 1187; (48) CCMA 1188; (49) CCMA 1189; (50) CCMA 1179; (51) CCMA 1180; (52) CCMA 1190; (53) CCMA 1185; (54) CCMA 1186; (55) CCMA1203; (56) CCMA 1279; (57) CCMA 1157; (58) CCMA 1278; (59) CCMA 1207; (60) CCMA 1277; (61) CCMA 1211; (62) CCMA 1208; (63) CCMA 1209; (64) CCMA 1210; (65) CCMA 1150; (66) CCMA 1153; (67) CCMA 1147; (68) CCMA 1148; (69) CCMA 1151; (70) CCMA 1152; (71) CCMA 1212; (72) CCMA 1200; (73) CCMA 1213; (74) CCMA 1214; (75) CCMA 1282; **Lactic acid bacteria (B):**(1) CCMA 1082; (2) CCMA 1083; (3) CCMA 1084; (4) CCMA 1085; (5) CCMA 1087; (6) CCMA 1089; (7) CCMA 1090; (8) CCMA 1105; (9) CCMA 1106; (10) CCMA 1108; (11) CCMA 1109; (12) CCMA 1111; (13) CCMA 1112; (14) CCMA 1115; (15) CCMA 1117; (16) CCMA 1120; (17) CCMA 1110; (18) CCMA 1058; (19) CCMA 1059; (20) CCMA 1064; (21) CCMA 1065; (22) CCMA 1067; (23) CCMA 1071; (24) CCMA 1072; (25) CCMA 1076; (26) CCMA 1078; (27) CCMA 1080.

In the Fig 3A the two principal components accounted for 44.15% of the total variability in the dataset (PC1 26.56% and PC2 18.59% of the total variance), showing discrimination between samples. The citric acid production was mainly characterized by treatments (16) *Bacillus pumilus* CCMA 1248; (17) *Bacillus pumilus* CCMA 1251; (28) *Bacillus horneckiae* CCMA 1265. Malic and tartaric production were associated with treatment (60) *Pantoea agglomerans* CCMA 1277. (57) *Arthrobacter koreensis* CCMA 1157 and (58) *Pantoea dispersa* CCMA 1278 were mainly characterized by propionic acid production. (70) *Microbacterium paraoxydans* CCMA 1152, (71) *Rhizobium radiobacter* CCMA 1212 and (72) *Rhizobium pusense* CCMA 1200 were mainly associated with succinic and acetic acid (Fig 3A). The all other treatments had balanced production of all the acids.

In Figure 3B two principal components accounted for 62.2% of the total variability in the dataset (PC1 38.96% and PC2 23.64% of the total variance). The citric acid production was mainly characterized by treatment (8) *Leuconostoc mesenteroides* CCMA 1105. (1) *Leu. mesenteroides* CCMA 1082 and (9) *Leu. mesenteroides* CCMA 1106 were associated with malic acid production and (21) *Lactobacillus plantatum* CCMA 1065 and (24) *Lact. plantatum* CCMA 1072 were characterized by lactic acid production. There was a formation of a group of treatments associated with the acetic acid production, such as made with *Leu. mesenteroides* (2) CCMA 1083, (4) CCMA 1085, (5) CCMA 1087, (7) CCMMA 1090, (11) CCMA 1109 and (13) CCMA 1112.

Lactic acid was the main acid produced in fermentations by lactic acid bacteria. Strains belonging to the *Lac. plantarum* increased the lactic acid concentration more than 50%. Strains that reduced or maintained the acetic and propionic acid concentration were also selected, because these acids in high concentrations (bigger than 1 $mg.ml^{-1}$), constitute negative deterioration factors of coffee as onion flavor and

interfere in a deprecatory way in final quality of the beverage (Lopez et al., 1989). The synthesis of the acid occurs through bacteria present on the surface of fruit and it can migrate to mucilage and pulp, which results in the organoleptic quality (Silva et al., 2013).

B. subtilis (CCMA 1234) and *B. pumilus* (CCMA 1251) were selected by the production of malic acid, and acetic and propionic acids reduction. *Cellulosimicrobium cellulans* (CCMA 1186) presented satisfactory succinic and citric acids production and propionic acid reduction. The specie *Pantoea dispersa* (CCMA 1203) was selected due to the high citric and succinic acids production and acetic acid reduction. *Microbacterium testaceum* (CCMA 1151) selected due to the high succinic and malic acid content produced, and acetic acid reduction.

High production of citric, succinic and lactic acid and low of acetic and propionic acids in a coffee pulp medium was used as selection factor to determine the best strain suitable to conduct coffee fermentation wet process. According to the results obtained by the acids production in coffee-based media, the selected strains selected for wet controlled fermentation were: *B. subtillis* CCMA 1234, *B. pumilus* CCMA 1251, *Cellulosimicrobium cellulans* CCMA 1186, *Pantoea dispersa* CCMA 1203, *Microbacterium testaceum* CCMA 1151, *Leu. mesenteroides* CCMA 1082 and CCMA 1105, *Lac. plantarum* CCMA 1065 and CCMA 1072.

3.2 *Controlled fermentation*

Mesophilic (five strains) and lactic acid bacteria (four strains) used as starter bacteria quantified by spread-plate during wet fermentation process (Fig. 4). Among mesophilic bacteria, there was a significant difference ($p<0.05$) among treatments and fermentation time (Fig 4A). During fermentation (T12 to T48) there was a significant

decrease ($p<0.05$) in population of mesophilic bacteria in almost all fermentations, except the one inoculated with *B. subtilis* CCMA 1234 and *B. pumilus* CCMA 1251, which showed an increase in population (from 4.91 to 5.34 and from 5.34 to 6.04 log CFU/g, respectively). The highest count of mesophilic bacteria (with 48h fermentation) was found in treatment with *C. cellulans* CCMA 1186 (7.43 log CFU/g) and with fermentations inoculated with acid lactic bacteria. The control presented 5.93 log CFU / g of total bacteria.

Acid lactic bacteria counting showed a significant difference ($p<0.05$) among treatments (Fig 4B). During fermentation (T12 to T48), there was a significant increase ($p<0.05$) in LAB population in fermentations inoculated with *B. subtilis* CCMA 1234, *B. pumilus* CCMA 1251, *C. cellulans* CCMA 1186, *M. testaceum* CCMA 1151 [reaching at the end of fermentation (48h) an average population of 6.65 log CFU/g]. Treatments inoculated with *P. dispersa* CCMA 1203, *Leu. mesenteroides* CCMA 1082, CCMA 1105 and *Lac. plantarum* CCMA 1065, CCMA 1072 was a decrease of acid lactic bacteria population [reaching at the end of fermentation (48h) with average population of 6.65 log CFU/g]. Largest population at end of fermentation (48h) was founded in treatment with *Lac. plantarum* CCMA 1065 (7.24 log CFU/g) and lowest population was treatment inoculated with *M. testaceum* (5.19 log CFU / g).

The population behavior in fermentation inoculated with mesophilic and lactic acid bacteria was the same, there was a decrease in population at end of the fermentation (Fig 4). However, in inoculated fermentations with lactic acid bacteria a larger population was observed (48h). This fact can be explained by it is possible anaerobic conditions or low oxygen concentration favor the presence of this group of bacteria, and the low pH value, which prevents the proliferation of other bacteria (Massawe and Lifa, 2010).

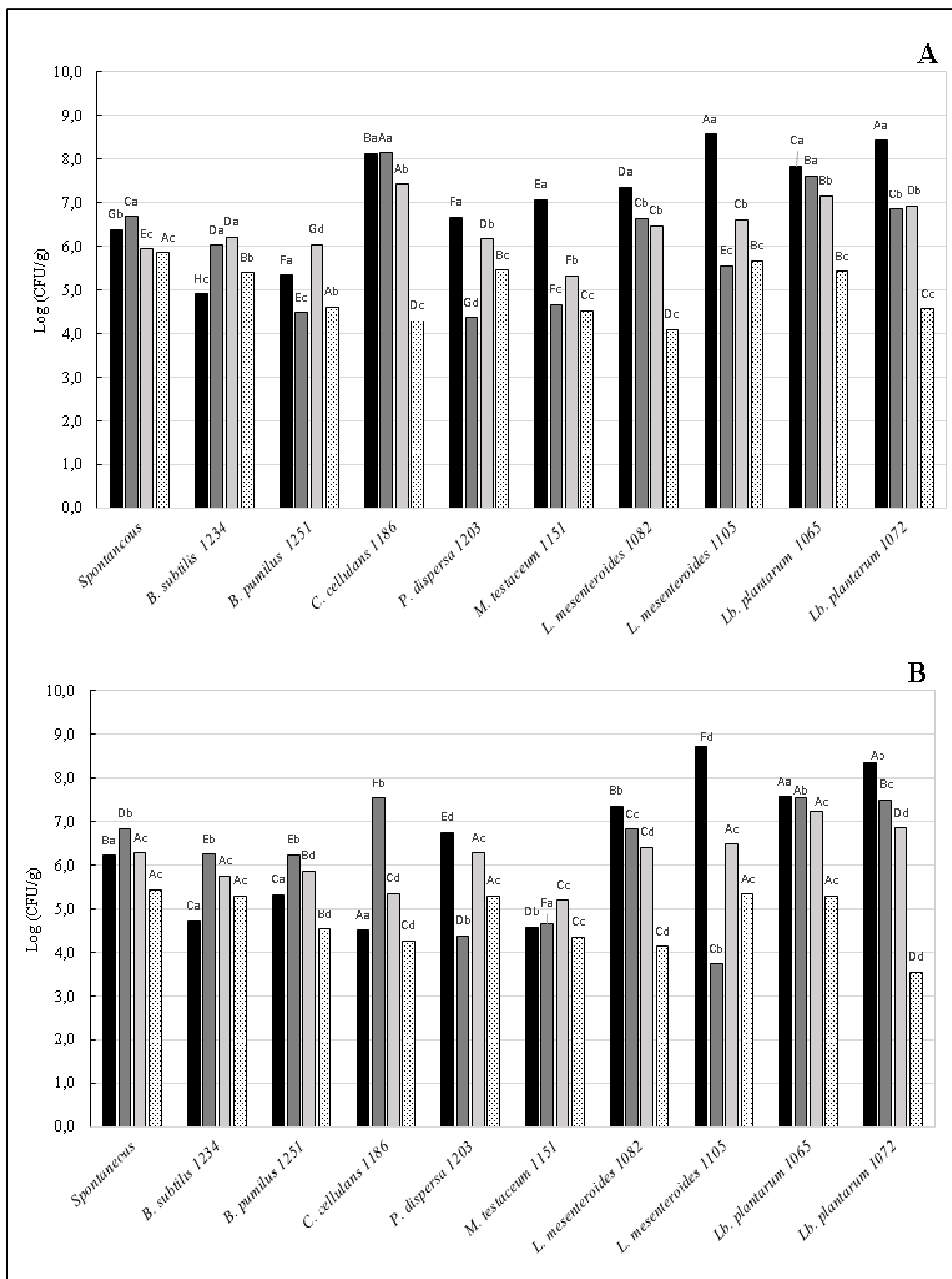

Figure 4. Mesophilic (A) and acid lactic (B) bacteria populations during the spontaneous and inoculated wet fermentation of coffee. Collected times: (■) T12 hours of fermentation; (■) T24 hours of fermentation; (□) T48 hours of fermentation; (⊡) dried coffee. Data are presented as mean. A-E mean values with different capital letters are significant at $p < 0.05$ for each treatment in the same time by Scott–Knott test. a-d mean values with different lowercase letter are significant at $p < 0.05$ for each time in the same treatment by Scott–Knott test.

Acids organics such as citric, malic, succinic, lactic, acetic, propionic, isobutyric of the fermented coffee and dried coffee were analyzed by HPLC (Fig. 5). Citric, propionic and isobutyric acids were not detected in any inoculated treatment and without inoculation. At zero time only malic and succinic acids were detected (0.17 and 0.25 $mg.g^{-1}$, respectively).

The organic acids production directly affects the fermentation and consequently the final quality of the coffee beverage. In this experiment succinic, lactic and acetic acids were detected and there was a significant difference ($p<0.05$) between treatments. During fermentation was a decrease in the succinic acid concentration (Fig 5A) over the times analyzed (12h, 24h and 48h of fermentation) inoculated with mesophilic bacteria and *Leu. mesenteroides* CCMA1082. Inoculated fermentations with lactic acid bacteria (*Leu. mesenteroides* CCMA 1105, *Lac. plantarum* CCMA 1065 and CCMA 1072) presented an increase in the succinic acid concentration. At end of drying (dried coffee) there was an increase in succinic acid concentration in all treatments. The fermentation with *Leu. mesenteroides* CCMA 1105 presented the highest concentration of this acid (0.54 $mg.g^{-1}$) and *Leu. mesenteroides* CCMA 1082 had the lowest concentration (0.22 $mg.g^{-1}$). Succinic acid may be produced by *Bacillus* spp. (Silva et al., 2013) and by heterofermentative lactic acid bacteria (Swiegers et al., 2005).

Lactic acid was a major acid quantified at end of fermentation in all treatments (Fig 5B). There was an increase ($p<0.05$) in lactic acid concentration, with the highest increase in final drying time (dried coffee). The fermentation inoculated with LAB showed the highest lactic acid concentration, which was already expected. The fermentation with mesophilic bacteria showed lactic acid final contractions (dried coffee) between 5.0 and 5.9 $mg.g^{-1}$, and fermentations with LAB between 7.6 and 8.1 $mg.g^{-1}$. Evangelista et al. (2015) also found that lactic acid was the main acid detected in

wet fermentations. Avallone and collaborators (2001) reported that degradation of mucilage present in this processing is due to the metabolism of lactic bacteria. The high concentration of lactic acid produced by them and consequent lowering of pH would cause the dissociation of the mucilage of the beans.

The highest acetic acid concentration of acetic acid was found in treatment inoculated with *P. dispersa* with 24 h of fermentation (0.29 $mg.g^{-1}$), followed by the control treatment (0.18 $mg.g^{-1}$) in 48h of fermentation. All treatments showed an increase in concentration of this acid at end of fermentation (48h). Spontaneous fermentation (control) and treatment inoculated with *C. cellulans* CCMA 1186 presented acetic acid concentrations (0.18 and 0.02 $mg.g^{-1}$, respectively) at end of drying (dried coffee). The fermentation inoculated with *Leu. mesenteroides* CCMA 1105 was not detected acetic acid at any time of sampling. The variation of acids during fermentation due to microbial metabolism changed the pH value and influences the microbiota present. The diffusion of theses acids into the bean could influence the final beverage flavor and quality (Evangelista et al., 2014a; Silva et al., 2013; Silva, 2015).

Acids variations during the fermentation occurs due to microbial metabolism and change the pH influencing the population of microorganism's present. These acids might positively or negatively alter the quality of the beverage, depending on the amount in which they are present. Citric and malic acids are already present in green coffee beans in a range of concentrations according mainly to the coffee variety, which may explain the low concentration of these acids found in this work. Citric acid can be degraded yielding mainly citraconic, glutaric, itaconic, mesaconic and succinic acids, while malic acid is responsible for the formation of fumaric and maleic acids (Clarke and Vitzthum, 2001). However, it is important to emphasize that only the presence of the acids does not influence the final quality, they must be present in concentrations that

interfere with the beverage, this was a criterion for the selection of the cultures. (Evangelista et al., 2015, Evangelista et al., 2014a, Silva et al., 2013, Silva, 2015).

A total of 44 volatile compounds were identified (presence and absence) by HS-SPME GC-MS analysis in the coffee fermentation without and with inoculation of bacteria (Table 3). Among these compounds, 37 were detected in initial fermentation and 32 were detected in end of fermentation. Acids, alcohols, aldehydes, ketones, esters, hidrocarbons, and other compounds (phenols, alkanes and alkaloids) were classified.

The volatile compounds predominantly found in initial fermentation belong to alcohols, aldehydes and ketones. Some compounds were detected in only one sample, such as 1-phenoxypropan-2-ol (*B. subtilis* CCMA 1234); phytol and ethyl linoleate (*C. cellulans* CCMA 1186); ethyl benzeneacetate (*P. dispersed* CCMA 1203); Octadecane (*L. mesenteroides* CCMA 1105). Spontaneous fermentation (control) and *Leu. mesenteroides* CCMA 1082 presented higher percentage of compounds of alcohol class (36 and 33%, respectively). Fermentations with *M. testaceum* CCMA 1151 and *Lac. plantarum* CCMA 1065 showed a higher percentage of aldehydes and ketones (30 and 33%, respectively).

At end of fermentation (48h) presented predominantly compounds belong to aldehydes and ketones. Some compounds were detected in only one or two samples, such as isovaleric acid (Spontaneous fermentation-control, *Leu. mesenteroides* CCMA 1105 and *Lac plantarum* CCMA1065); 2, 3 –butanediol (*C. cellulans* CCMA1186 and *P. dispersa* CCMA1203); phenethyl alcohol (Spontaneous fermentation- control, *Leu. mesenteroides* CCMA 1105 and *Lac plantarum* CCMA1065); β -linalool (*P. dispersa* CCMA1203 and *Leu. mesenteroides* CCMA 1105); 2-hexenal (Spontaneous fermentation-control and *Leu. mesenteroides* CCMA 1105); ethyl linoleate (*P. dispersa*

CCMA1203); ethyl 2-hydroxypropanoate (Control and *Leu. mesenteroides* CCMA 1105); tetracosane (*C. cellulans* CCMA1186 and *Leu. mesenteroides* CCMA 1105).

Some volatile compounds detected in this study are described in the literature by providing aromatic notes such as caramel, nutty, burnt, buttery, floral, and almond (Table 4). The main groups (acids, alcohols, aldehydes and ketones) can be correlated with contributed to the citric and herbaceous buttery, caramel-like, musty, mushroom like, or fruity flavors that were desirable on coffee beverage (Flament, 2002; Czerny and Grosch, 2000; Gonzalez-Rios et al., 2007).

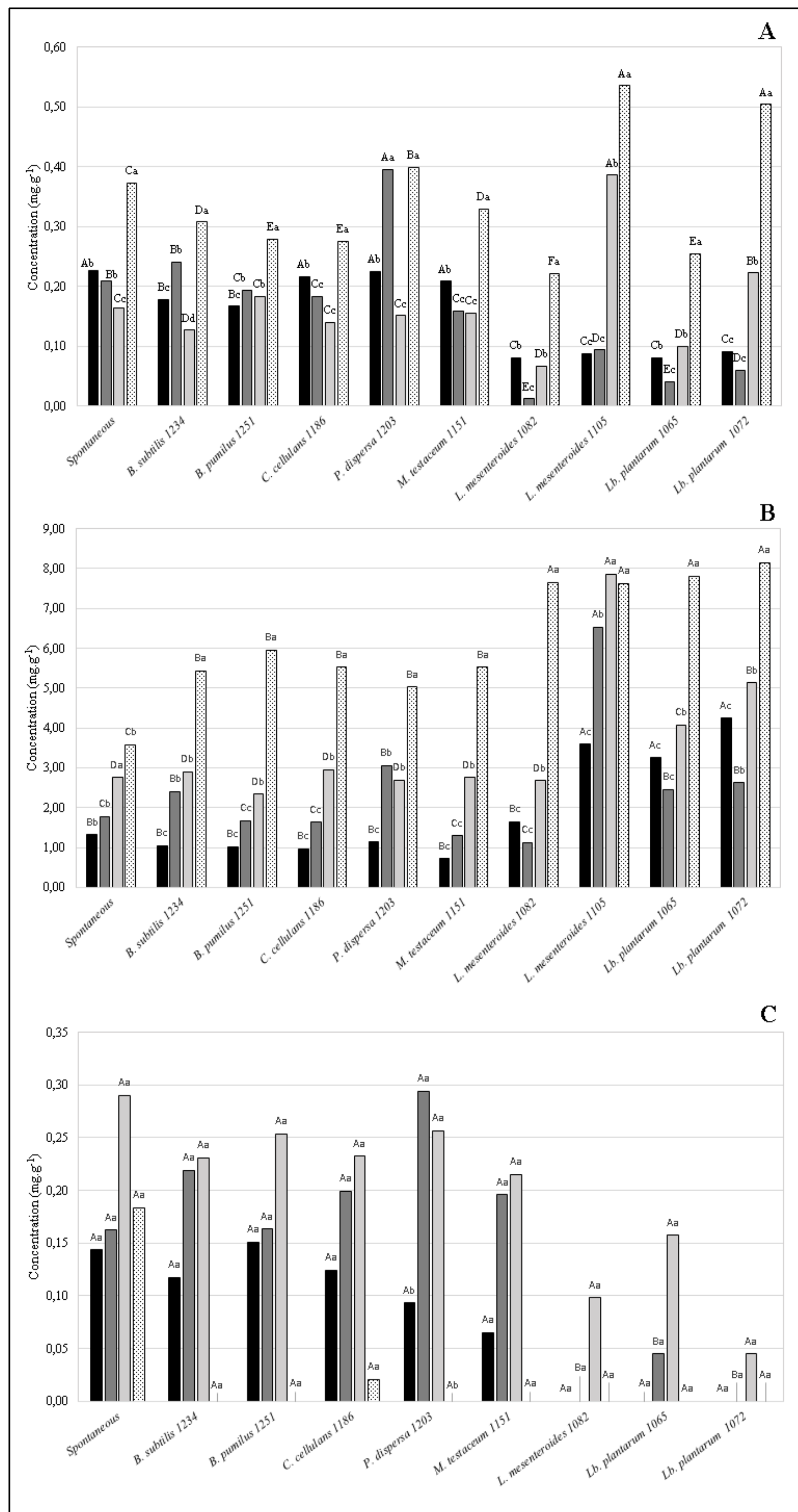

Figure 5. Effects of inoculation with different strains on organic acids concentration ($mg.g^{-1}$) present in wet coffee fermented. A) Succinic acid; B) Lactic acid and C) Acetic acid. Collected times: (■) T12 hours of fermentation; (■) T24 hours of fermentation; (□) T48 hours of fermentation; (⚃) dried coffee. Data are presented as mean. A-I mean values with different capital letters are significant at $p < 0.05$ for each treatment in the same time by Scott–Knott test. a-d mean values with different lowercase letter are significant at $p < 0.05$ for each time in the same treatment by Scott–Knott test.

Table 3. Volatile compounds identified by Headspace—Solid Phase Microextraction—Gas Chromatography Mass Spectrometry (HS–SPME GC–MS) in spontaneous and inoculated wet fermentation.

Compounds	Treatments*																			
	A	A_1	B	B_1	C	C_1	D	D_1	E	E_1	F	F_1	G	G_1	H	H_1	I	I_1	J	J_1
Acids																				
Acetic acid	■		■		■		■						■						■	
Isolvaleric acid	■	■	■	■	■		■						■		■	■			■	■
Hexadecanoic acid	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■
Tetradecanoic acid	■	■	■		■		■	■		■				■	■	■		■		■
Hexanoic acid		■		■		■		■		■		■		■				■		■
Octanoic acid		■		■		■				■		■		■		■				
Alcohols	A	A_1	B	B_1	C	C_1	D	D_1	E	E_1	F	F_1	G	G_1	H	H_1	I	I_1	J	J_1
1-Hexadecanol	■					■			■	■	■	■	■	■		■		■	■	
2-Heptanol	■																			
2,3-Butanediol	■				■		■	■		■	■		■							
5-methyl-2-hexanol	■								■				■						■	
Benzyl alcohol	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
1-phenoxypropan-2-ol			■																	
Phenethyl alcohol	■	■	■		■		■		■		■		■		■	■	■		■	■
Pentadecanol			■			■		■	■	■		■	■	■		■		■	■	
Tetradecanol	■				■		■												■	
Tridecanol			■										■							
Dodecanol						■								■		■				
β-linalool	■		■		■				■	■	■		■		■	■	■		■	
Phytol							■													
Aldehydes and Ketones	A	A_1	B	B_1	C	C_1	D	D_1	E	E_1	F	F_1	G	G_1	H	H_1	I	I_1	J	J_1
Acetoin		■								■	■		■		■	■				■
Benzaldehyde	■	■	■		■		■		■	■	■	■	■	■	■	■	■		■	■
Pentadecanal	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
2-Hexenal		■	■													■	■		■	
9,17-Octadecadienal, (Z)-	■		■								■				■		■			

(continue…)

(conclusion of table 3).

Compound	A	A_1	B	B_1	C	C_1	D	D_1	E	E_1	F	F_1	G	G_1	H	H_1	I	I_1	J	J_1
2-Pentadecanone, 6,10,14-trimethyl-	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
(E)-Geranyl acetone					■	■					■	■	■	■	■	■				
2,4-Hexadienal, (E,E)-	■	■					■	■								■				
Esters	A	A_1	B	B_1	C	C_1	D	D_1	E	E_1	F	F_1	G	G_1	H	H_1	I	I_1	J	J_1
Methyl hexadecanoate	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Ethyl hexadecanoate	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Diisobutyl phthalate	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Ethyl 2-hydroxypropanoate		■														■				
Ethyl linoleate							■	■		■										
Ethyl benzeneacetate									■											
Hydrocarbons	A	A_1	B	B_1	C	C_1	D	D_1	E	E_1	F	F_1	G	G_1	H	H_1	I	I_1	J	J_1
Heptadecane		■	■	■		■	■	■	■	■	■	■	■			■			■	
Hexadecane		■		■		■	■	■	■	■		■	■	■		■	■	■	■	■
Hexacosane			■				■		■											
Tetracosane							■	■							■			■		
Octadecane				■				■		■				■	■			■		
Others**	A	A_1	B	B_1	C	C_1	D	D_1	E	E_1	F	F_1	G	G_1	H	H_1	I	I_1	J	J_1
Methyl salicylate	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Vanillin	■		■		■	■	■		■		■	■		■	■		■	■	■	
Heneicosane	■	■	■	■	■		■	■	■	■	■	■	■	■	■	■	■	■		■
Caffeine	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■

*Treatments: Spontaneous fermentation (A); *B. subtilis* CCMA1234 (B); *B. pumilus* CCMA1251 (C); *C. cellulans* CCMA1186 (D); *P. dispersa* CCMA1203 (E); *M. testaceum* CCMA1151 (F); *Lec. mesenteroides* CCMA1082 (G); *Lec. mesenteroides* CCMA1105 (H); *Lac plantarum* CCMA1065 (I); *Lac. plantarum* CCMA1072 (J). Capital letters without number: beginning of fermentation; Capital letters with subscript number: end of fermentation **Inclued: Alkaloids.

5 Conclusion

The results obtained in this study showed that the use starter bacteria in wet coffee fermentation is an alternative to producing coffees with distinct quality and aromas. Bacteria play an important role in the fermentation and production of metabolites. It is possible to direct the production of flavor precursors using distinct strains of bacteria. *Pantoea dispersa* CCMA 1203; *C. cellulans* 1186, *Leu. mesenteroides* CCMA 1105 and *Lac. plantarum* CCMA 1065 gave a better result in relation to acid and volatile compounds production in green coffee. Future work should be conducted to evaluate these strains in different coffee varieties and in co-culture and the sensory profile generated using these strains.

Acknowledgments

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ANEXO 1. Material complementar referente ao artigo 2.

Additional materials.

Table 1. Identification of volatile compounds (GC-MS peak areas) in green coffee of Ouro Amarelo, Mundo Novo and Catuaí Vermelho varieties and odor description of compounds.

Compounds	LRI		GC – MS peakarea (10^4)									Odor description[a]
			Ouro Amarelo			Mundo Novo			Catuai Vermelho			
	FFAP[b]	Literat.	Fermentation		DC	Fermentation		DC	Fermentation		DC	
			PC	DMC		PC	DMC		PC	DMC		
Acids												
Hexanoicacid	1853	1845	0,7	1,0	3,3	0,8	0,8	3,9	0,6	0,6	4,6	Sour, fatty, sweaty, cheese-like
Heptanoicacid	1960	1942	n.d.	n.d.	0,7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	Fatty, nuity, fruity
Octanoicacid	2068	2050	0,1	0,4	0,9	0,3	0,4	0,9	0,2	0,3	0,9	Fatty, waxy, cheese-like
Nonanoicacid	2176	2165	n.d.	0,5	0,6	0,5	0,7	1,4	0,3	0,5	0,5	Waxy, cheese-like
Tridecanoicacid	2695	2724	n.d.	n.d.	n.d.	n.d.	1,3	1,6	1,1	0,7	2,9	-
Alcohols												
2-Buten-1-ol, 3-methyl-	1326	1334	2,8	1,6	1,5	n.d.	2,3	2,4	2,1	2,7	0,4	Fresh, herbacous-fruity-green, lavender-like
1-Hexanol	1357	1358	13,3	4,1	25,9	7,8	5,8	14,0	7,5	6,3	23,8	Winey, fatty, fruity
2-Hexen-1-ol, (Z)-	1413	1403	0,7	1,5	n.d.	0,9	2,3	n.d.	1,5	3,3	n.d.	
1-Octen-3-ol	1458	1462	17,8	6,2	20,4	8,8	8,7	28,9	11,9	18,0	19,4	Earthy, green, mushroom-like
1-Heptanol	1462	1460	n.d.	n.d.	9,0	n.d.	n.d.	5,4	n.d.	n.d.	4,0	Green, fatty, dairy, lactonic
2-Ethyl-1-hexanol	1492	1492	n.d.	n.d.	1,8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	Fermented, yeast, fusel, ethereal
2,3-Butanediol, [R-(R*,R*)]-	1550	1544	n.d.	n.d.	1,6	n.d.	n.d.	1,2	n.d.	n.d.	3,9	Fruity, creamy, buttery
1-Octanol	1564	1559	2,9	1,3	15,5	1,0	0,8	11,3	0,5	0,6	7,5	Green, fatty, coconut
2,3-Butanediol, [S-(R*,R*)]-	1588	1581	0,7	0,8	6,0	6,7	6,2	6,4	4,6	0,8	10,7	-
1-Nonanol	1667	1665	0,6	0,5	1,7	0,4	0,3	2,5	0,4	0,3	3,3	-
2-Heptanol	1324	1312	185,1	63,2	15,4	53,2	35,4	13,5	74,4	74,1	3,4	Fresh, lemon-like, grassy-herbaceous, sweet-floral, green, fatty, nutty, sweet

(continue..)

Benzylalcohol	1882	1882	3,4	1,4	1,0	3,6	1,8	1,3	2,3	0,9	0,7	Floral, rose-like, phenolic, balsamic
2-Phenylethanol	1914	1902	14,8	12,6	4,4	15,5	10,7	10,1	15,2	11,5	2,9	Floral, rose-like, honey
Aldehydes												
2-Pentenal, (E)-	1153	1127	1,3	0,9	1,7	1,3	2,2	1,7	0,9	2,5	0,9	-
3-methyl-2-Butenal	1207	1212	8,6	4,0	3,5	n.d.	5,1	5,0	6,1	10,7	1,5	Green, bitteralmond, furfurylic
Octanal	1287	1303	0,7	n.d.	8,7	n.d.	n.d.	7,9	n.d.	n.d.	5,8	Green, fatty, orange, juicy.
2-Heptenal, (E)-	1317	1321	1,5	0,9	5,0	2,4	2,8	10,7	1,6	1,2	6,2	Pungent, green, fatty, vegetable-like, apple-like
Nonanal	1393	1416	8,2	4,0	60,1	n.d.	5,1	70,0	6,3	3,7	63,0	Strong, soap-like, metallic
2-Octenal	1426	1429	12,8	6,3	9,0	17,0	12,0	22,5	7,4	7,2	14,8	Fatty-nutty, fatty-fruity, green
2,4-Heptadienal, (E,E)-	1466	1497	5,8	4,4	0,9	4,5	3,8	1,7	5,5	3,7	0,7	Fatty, woody, herbal
Decanal	1498	1500	n.d.	n.d.	4,4	n.d.	n.d.	19,4	n.d.	n.d.	15,2	-
2-Nonenal	1532	1533	2,6	2,5	5,6	5,0	2,9	11,3	2,3	3,7	9,4	Fatty, cardboard-like
2,4-nonadienal (E,E)	1699	1701	1,3	0,9	2,7	2,2	1,1	9,2	1,3	0,8	5,9	Fatty-soap, geranium-like, metallic
2-Undecenal	1747	1755	0,5	0,9	1,4	0,8	0,5	1,4	0,6	0,3	0,9	-
Tetradecanal	1920	1930	0,3	0,2	0,6	0,3	0,2	0,5	1,1	0,2	0,5	-
Pentadecanal	2026	2042	0,9	0,3	0,4	0,5	0,4	1,1	4,2	0,6	0,9	-
2-Furaldehyde	1473	1433	n.d.	n.d.	1,1	n.d.	n.d.	2,0	n.d.	n.d.	1,1	Almond-like, woody, bready
Benzaldehyde	1520	1506	37,3	24,6	13,2	37,0	34,8	33,6	44,3	32,5	7,2	Bitter almond, cherry-like, sweet
3-methyl-benzaldehyde	1616	1623	n.d.	n.d.	0,5	n.d.	n.d.	0,7	n.d.	n.d.	0,5	-
Esters												
Methylbenzeneacetate	1759	1754	1,2	0,8	n.d.	2,1	1,1	n.d.	1,6	0,9	n.d.	Honey, fruity
Dodecanoic acid methylethylester	1838	1831	0,3	n.d.	n.d.	n.d.	0,2	0,4	0,2	0,2	1,1	-
Methyl-2-hydroxybenzoate	1766	1765	1,2	1,1	5,3	2,4	0,9	2,0	2,9	4,3	0,5	-
1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester	2540	2540	5,4	3,5	8,8	3,4	10,5	23,2	2,4	1,5	27,1	-

(continue...)

(conclusion of table 4)

Hydrocarbons												
Tetradecane	1393	1400	12,3	12,8	21,0	17,7	20,3	27,5	11,7	10,6	11,0	-
1-Tetradecene	1439	1454	3,7	5,2	n.d.	8,3	3,1	n.d.	1,5	4,5	n.d.	-
Pentadecane	1496	1500	40,8	17,8	6,3	20,1	10,8	5,1	6,6	8,5	3,8	-
Heptadecane	1705	1700	0,6	0,4	0,6	0,4	0,4	0,5	0,3	0,3	0,9	-
1,4-dimethyl-benzene	1156	1119	4,3	2,2	2,4	2,8	3,2	2,1	0,9	5,8	3,3	Pungent, grassy, querosene-like
Ketones												
2-Heptanone	1190	1158	5,6	0,7	5,1	2,7	1,1	5,3	6,1	1,8	4,4	-
2-Octanone	1282	1309	n.d.	n.d.	1,9	n.d.	n.d.	3,9	n.d.	n.d.	2,3	Floral, bitter-green, musty-herbaceous, unripe apple
2-Nonanone	1388	1403	n.d.	n.d.	1,6	n.d.	n.d.	2,1	n.d.	n.d.	0,9	Fruity-floral, fatty, herbaceous
6-Methyl-3,5-heptadiene-2-one	1592	1582	n.d.	n.d.	4,6	n.d.	n.d.	7,4	n.d.	n.d.	3,8	-
Lactones												
2(3H)-Furanone	1910	1910	n.d.	n.d.	1,5	n.d.	n.d.	0,8	n.d.	n.d.	1,0	-
Dihydro-5-pentyl-2(3H)-Furanone	2022	1998	n.d.	n.d.	1,6	n.d.	n.d.	2,2	n.d.	n.d.	1,6	Coconut, sweet, fatty-milk
Furans												
2-pentyl-furan	1222	1231	11,8	10,7	24,9	18,2	20,2	87,4	9,3	23,2	49,2	Fruity, green, earthy, beany
Phenols												
Phenol	2013	1996	n.d.	n.d.	0,5	n.d.	n.d.	0,6	n.d.	n.d.	0,7	Phenolic, rubbery, plastic-likemedicinal smoky, shoe-polish-like
Pyrazines												
Pyrazine	1523	1520	23,2	12,7	15,5	13,1	15,9	13,7	19,6	15,9	16,6	Pungent, sweet
Terpenes												
Beta-linalool	1555	1555	27,8	13,2	1,8	21,6	14,0	4,4	16,9	15,2	0,4	Sweet, flowery

n.d.: not detected; LRI: Linear Retention Indices obtained from references or literature values. [a]Odor descriptions obtained from Czern and Grosch (2000); Lee et al. (2016a, 2016b); Flament (2002); [b]FFAP: GC - Free fatty acid phase column. PC: pulped coffee; DMC: demucilled coffee; DC: dried coffee

Table 5. Identification of volatile compounds (GC-MS peak areas) in roasted coffee (RC) of Ouro Amarelo, Mundo Novo and Catuaí varieties and odor description of compounds.

Compounds	LRI		GC – MS peakarea (10^4)			Odor description[a]
	FFAP	Literat.	Ouro Amarelo	Mundo novo	Catuai vermelho	
Acids						
Tridecanoicacid	2695	2724	2,5	2,6	6,2	-
Alcohols						
2-Phenylethanol	1914	1902	19,7	22,0	19,0	Flora, rose-like, honey
Aldehyde						
2-methyl-2-butenal	1134	1090	8,8	4,6	5,5	Fruity-apple, estery, banna, pear
Benzaldehyde	1520	1525	27,9	22,2	22,6	Bitter almond, cherry-like, sweet Cherry, pistachioflavor
Hydrocarbons						
Tetradecane	1404	1400	59,6	65,6	48,4	-
Dodecane	1203	1200	41,6	27,8	34,0	-
Ketones						
4-Heptanone	1154	1145	17,6	9,2	10,7	Etheral-fruity, pungente, pineapple, strawberry
3-Penten-2-one	1152	1127	22,7	12,8	13,9	Nutty, fruity, green-leaf, floral
1-hydroxy-2-Butanone	1374	1380	429,7	168,9	269,5	-
3-hydroxy-2-butanone	1283	1268	455,3	180,7	256,6	Buttery, creamy, dairy-like
2-Cyclopenten-1-one	1351	1345	27,2	12,9	16,8	-
2-Methyl-2-cyclopentenone	1363	1366	30,2	15,2	19,3	-
2,3-Dimethyl-2-cyclopenten-1-one	1533	1550	42,8	24,8	29,1	Mouldy, dirty
3-Ethyl-2-hydroxy-2-cyclopenten-1-one	1897	1924	88,4	57,4	69,9	Caramel, sweet, sugary, maple

(continue…)

1-(2-hydroxy-5-methylphenyl)-Ethanone	2201	2178	455,3	667,6	442,2	-
Esters						
bis(2-methylpropyl) ester- 1,2-benzenedicarboxylic acid	2540	2548	17,5	24,6	7,9	-
Ethylenepropionate	1609	1630	179,3	98,3	127,7	-
Phenols						
o-Guaiacol	1864	1853	117,4	97,9	89,8	Phenolic, spicy, smoky, woody, burnt
p-Ethylguaiacol	2033	2037	56,9	71,8	49,3	Spicy
Furaldehydes						
5-Ethyl-2-furaldehyde	1632	1634	36,3	29,4	29,6	-
5-Acetoxymethyl-2-furaldehyde	2208	2199	51,3	36,8	45,6	-
5-Hydroxymethyl furfural	2516	2512	17,7	17,9	15,3	Herbaceous, winey-ethereal
Furans						
2,3-dihydrobenzofuran	2405	2389	13,1	21,2	11,9	Styrene-like
2-Vinylfuran	1124	1063	23,3	12,6	15,1	Ethereal, rum, cocoa note
Furfurylmethylether	1244	1251	29,4	12,8	15,9	Nutty, coffee grounds-like, rich, phenolic
Difurfurylether	1991	1977	97,8	87,5	81,6	Coffee-like, toastedodour
Lactones						
Dihydro-2(3H)-Furanone	1755	1726	232,7	127,1	169,3	Faint, sweet-aromatic, buttery, acrid, rubbery
5-acetyldihydro-2(3H)-Furanone	2071	2096	95,0	61,5	72,0	-
2,5-Dimethyl-4-hydroxy-3(2H)-furanone	2043	2014	137,4	110,9	117,4	Caramel, rasty, sweet

(continue…)

Pyrans						
3-Hydroxy-2-methyl-4-pyrone	1969	1943	359,4	312,7	296,7	Warm-fruity, caramellic-sweet, pineapple jam, strawberry jam
Pyrazines						
Pyrazine	1217	1241	319,3	170,3	201,8	Pungent, sweet
2,3-Dimethylpyrazine	1343	1357	286,0	165,8	197,3	Nutty, coffee-like, caramellic, cocoa-like
2-Propylpyrazine	1415	1428	38,1	24,8	24,8	Green vegetable, burnt
2,6-Diethylpyrazine	1431	1432	94,4	75,3	82,8	Hazelnut-like
2,5-Diethylpyrazine	1455	1449	19,7	16,3	15,5	Hazelnut-like
Ethenylpyrazine	1437	1434	68,7	51,4	52,0	Nutty, woody, roasty, cocoa-like, green
3-Ethyl-2,5-dimethylpyrazine	1444	1459	487,4	375,0	462,2	Hazelnut-like, earthy, baked, potato-like
2-Ethyl-3,6-dimethylpyrazine	1459	1449	159,1	136,3	139,6	-
3,5-diethyl-2-methylpyrazine	1492	1509	78,8	71,0	79,2	Coffee-like, green, nuts
2-Isoamyl-6-methylpyrazine	1462	1500	32,0	27,3	26,6	-
2-Methyl-5-vinylpyrazine	1495	1505	79,3	63,9	62,7	Frash, hazelnut, earthy
2-Acetyl-1-methylpyrrole	1655	1645	129,9	104,4	112,7	Nutty, floral, fruity
1-(6-Methyl-2-pyrazinyl) ethanone	1693	1679	199,5	177,2	175,1	Popcorn-like
Benzoparadiazine	1894	1905	13,4	13,7	12,5	-
Pyridines						
3-Methylpyridine	1297	1285	25,1	7,9	12,4	Green, earthy, hazelnut-like

(continue…)

(conclusion of table 5)

Pyrroles						
1-Methylpyrrole	1160	1142	79,7	51,7	52,9	Smoky-tarry, woody-herbaceous
3-Methylpyrrole	1561	1569	17,9	14,6	12,3	-
1-Methylpyrrole-2-carboxaldehyde	1618	1620	435,3	274,0	168,8	Cracker-popcornlike
1-Furfurylpyrrole	1833	1817	378,7	343,5	320,3	Hay-like, mushroom-like, green
2-Acetylpyrrole	1976	1959	405,9	326,2	345,0	Musty, nutty, coumarin-like
2-Pyrrolylcarboxaldehyde	2027	2006	394,0	328,5	341,2	Corny, pungent, beefy, coffee-like
Indole	2442	2435	32,5	47,1	28,3	Floral notes
Terpene						
Beta-myrcene	1173	1152	8,8	5,1	4,6	-
Thiazoles						
4-Methylthiazole	1280	1279	24,6	16,2	16,9	Tomato, fruity, nutty, green, meaty
Thiophenes						
3-Methylthiophene	1131	1110	7,5	4,3	4,5	Fatty, winey

n.d.: not detected; LRI: Linear Retention Indices obtained from references or literature values. [a]Odor descriptions obtained from Czern and Grosch (2000); Lee et al. (2016a, 2016b); Flament (2002); [b]FFAP: GC - Free fatty acid phase column.

ANEXO 2. Dendogramas gerados pelo MALDI- TOF referentes ao artigo 2.

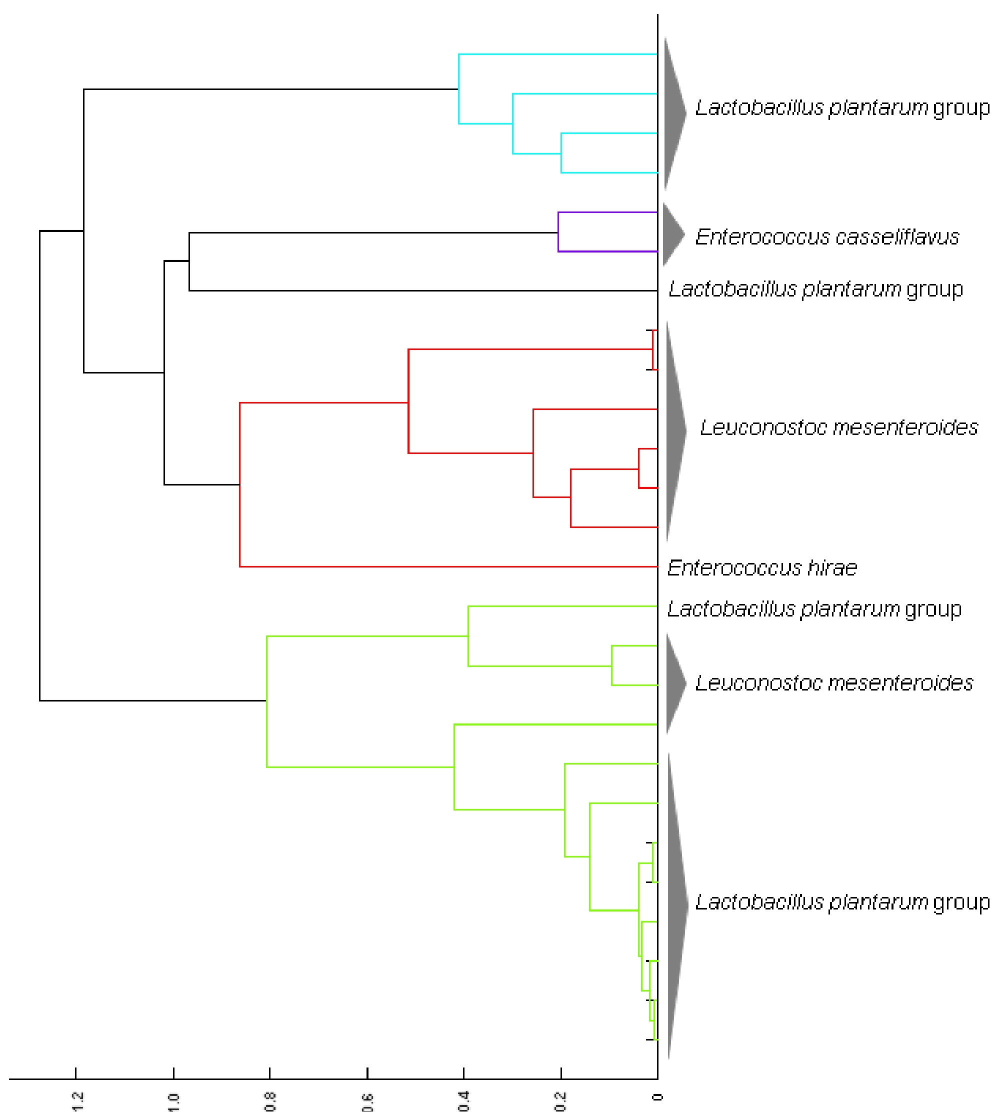

Figura 1. Dendograma das espécies de bactérias láticas isoladas na variedade Ouro Amarelo

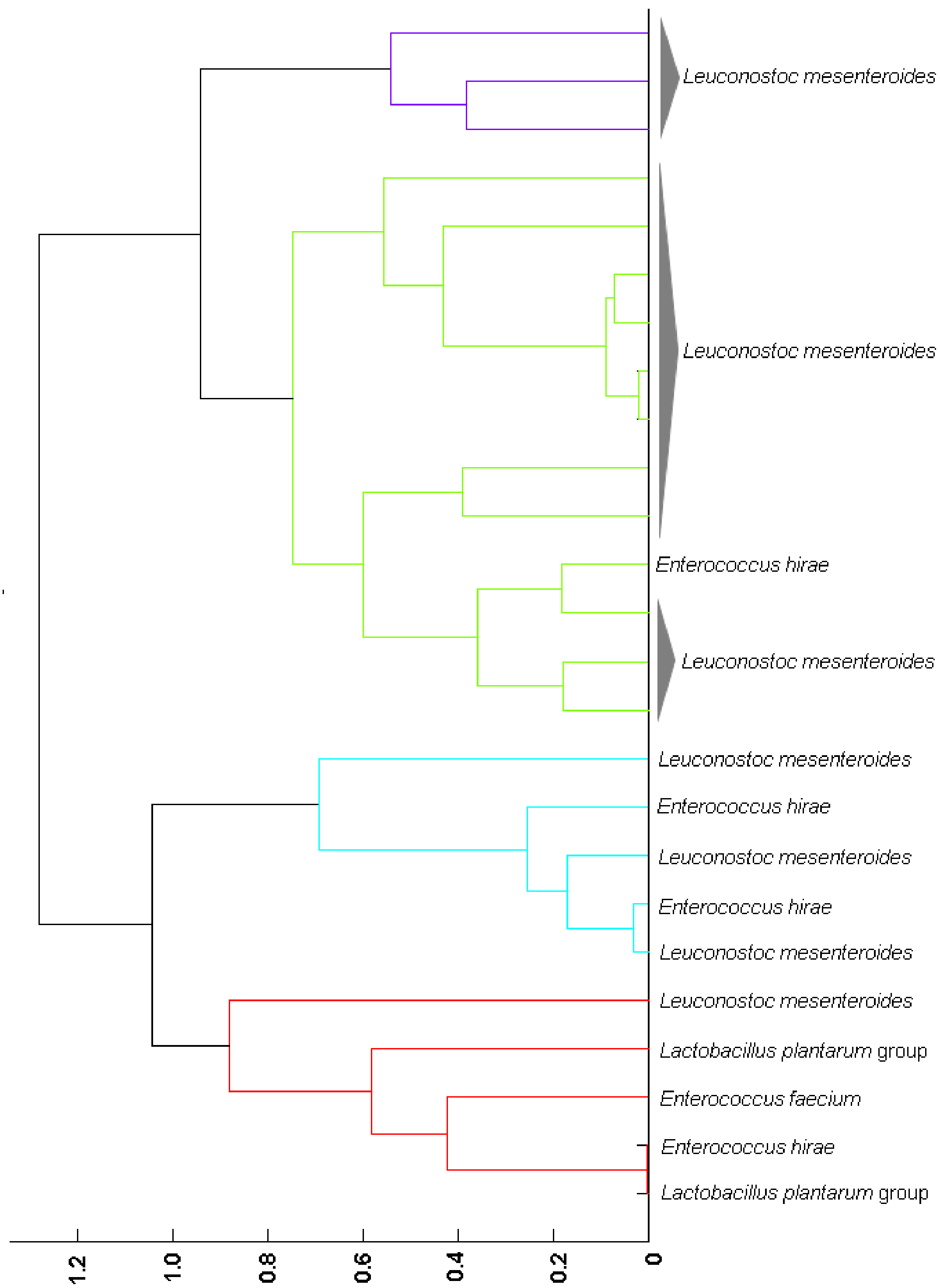

Figura 2. Dendograma das espécies de bactérias láticas isoladas na variedade Mundo Novo.

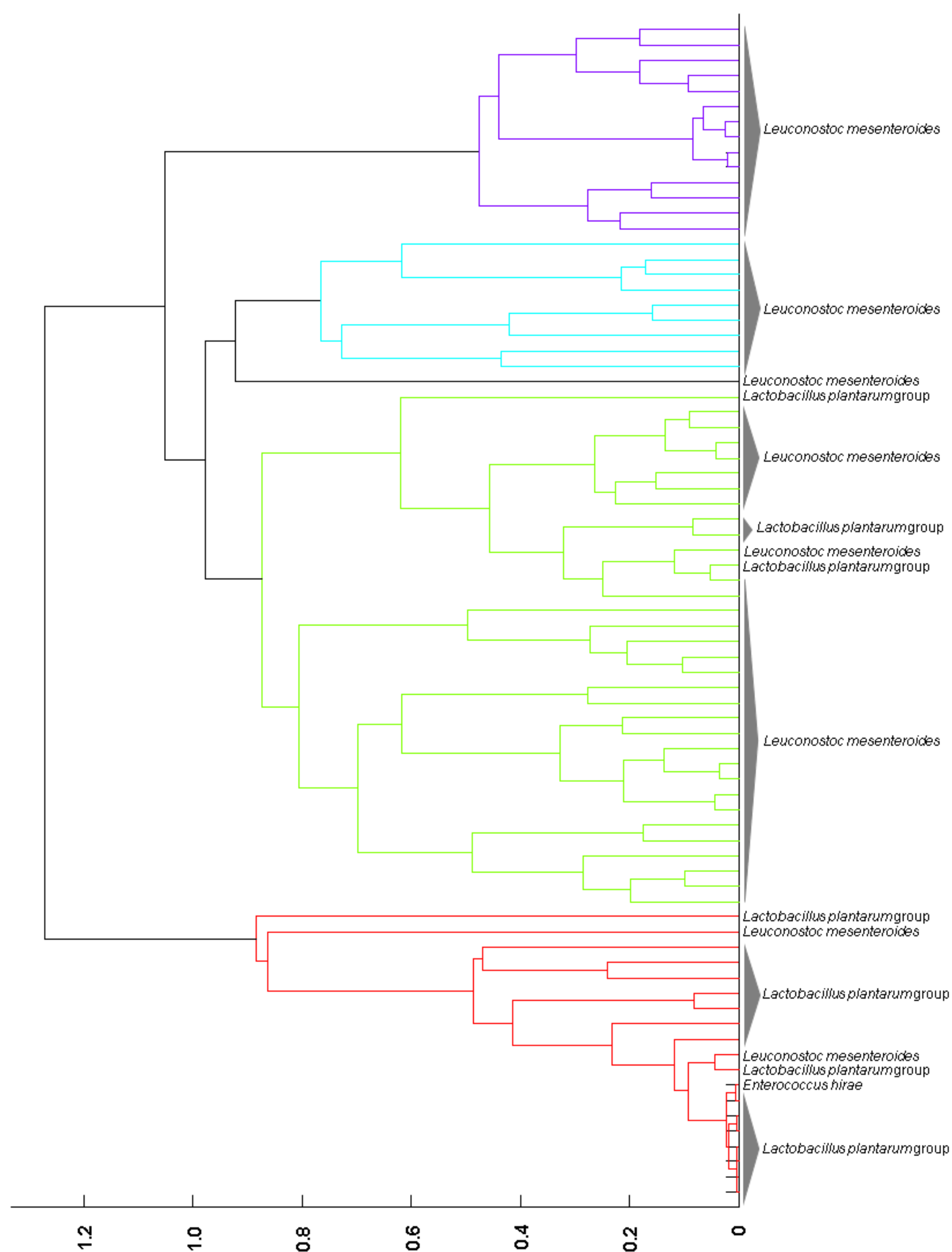

Figura 3. Dendograma das espécies de bactérias láticas isoladas na variedade Catuaí Vermelho.

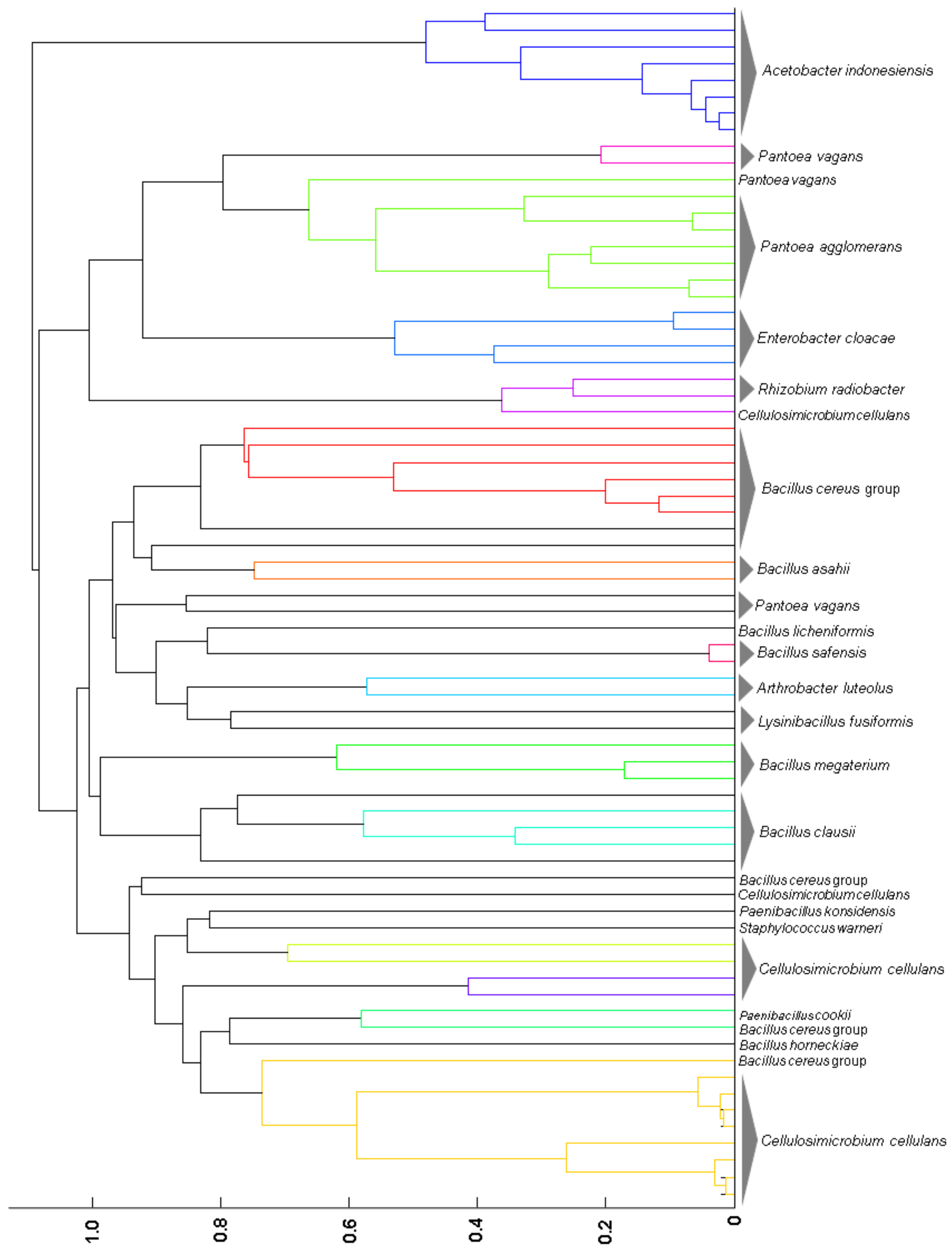

Figura 4. Dendograma das espécies de bactérias mesofílicas isoladas na variedade Ouro Amarelo.

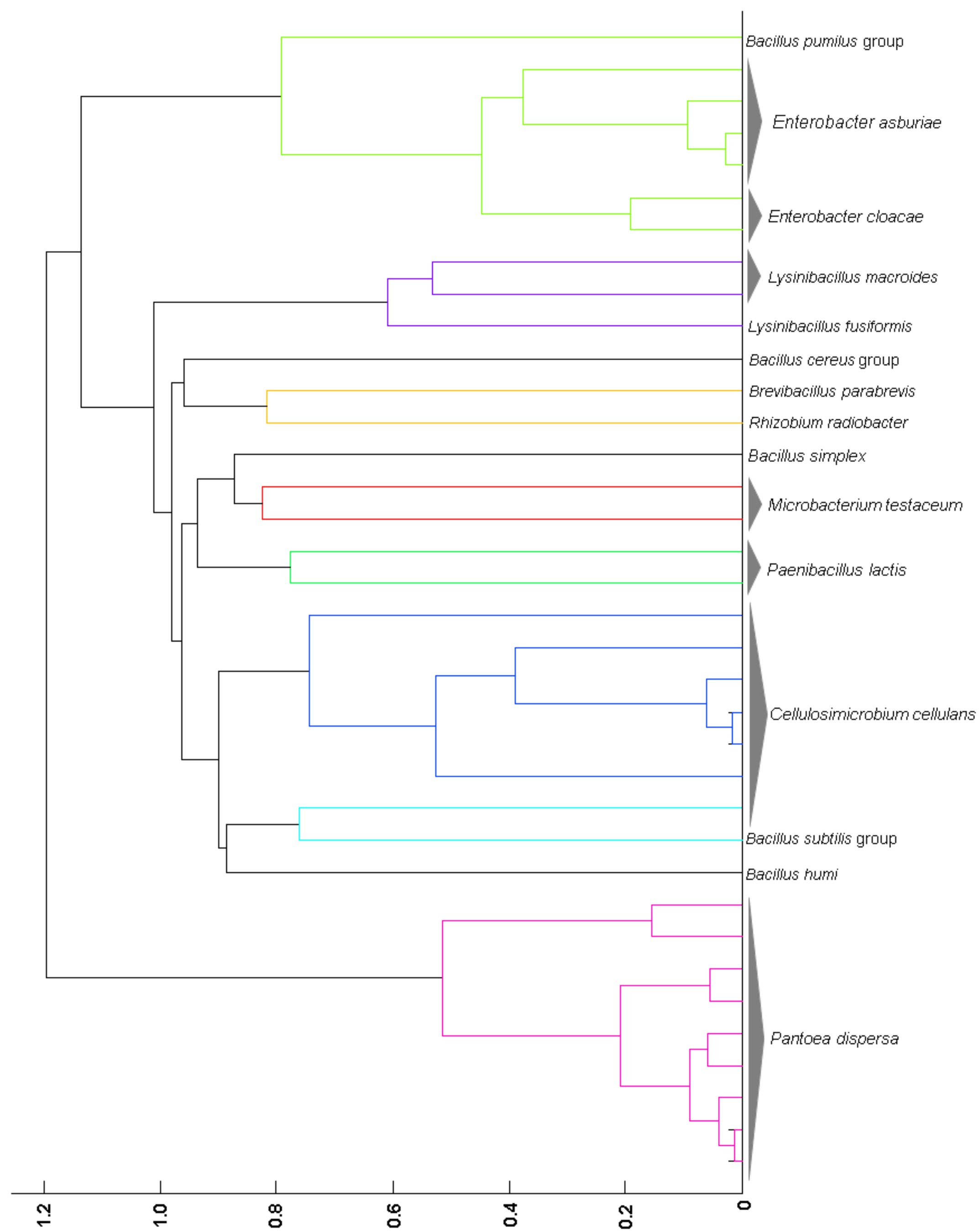

Figura 5. Dendograma das espécies de bactérias mesofílicas isoladas na variedade Mundo Novo.

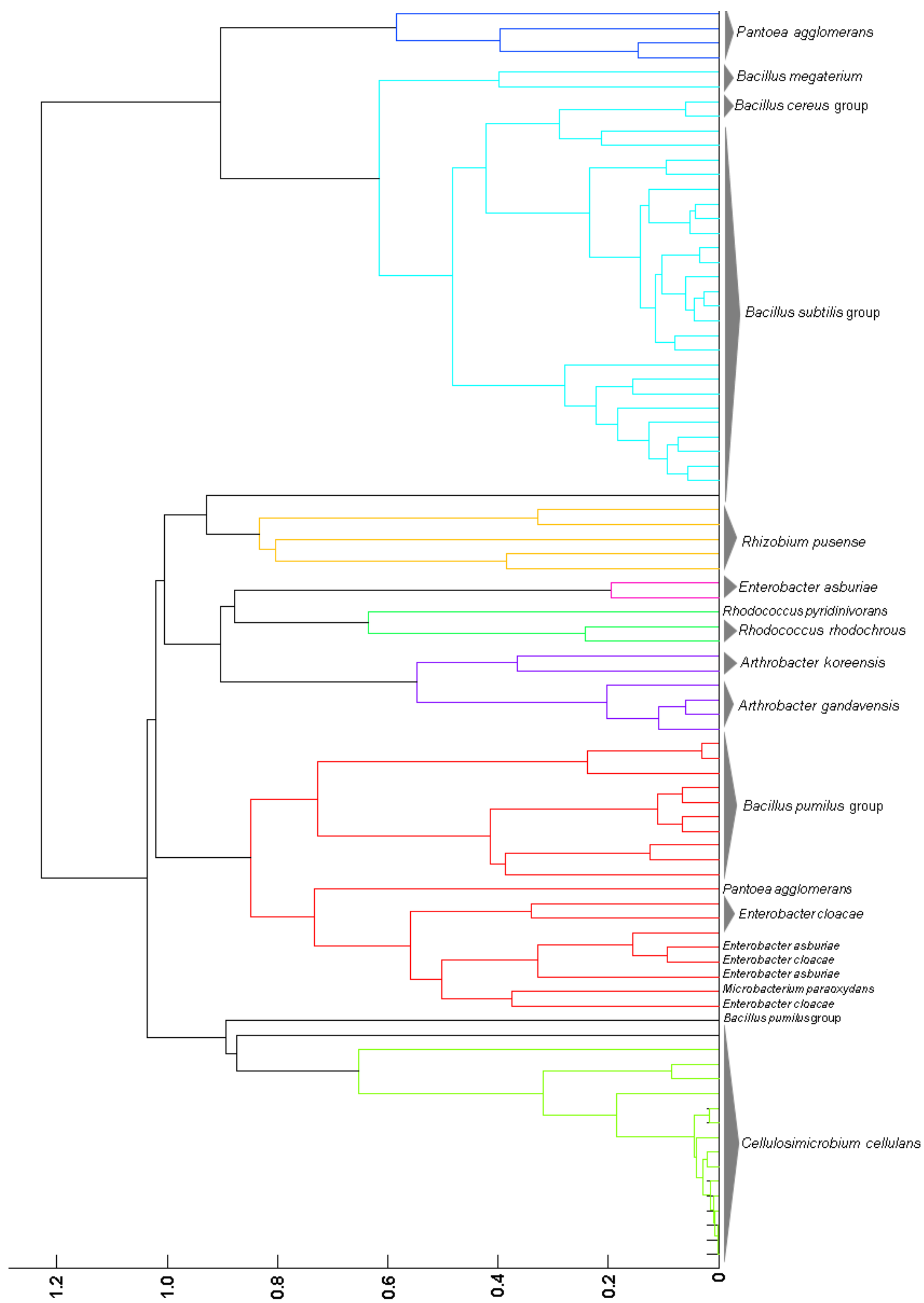

Figura 6. Dendograma das espécies de bactérias mesofílicas isoladas na variedade Catuaí Vermelho.

ANEXO 3. Cromatogramas gerados pelo programa LCSolutions GC-MS referentes ao Artigo 3.

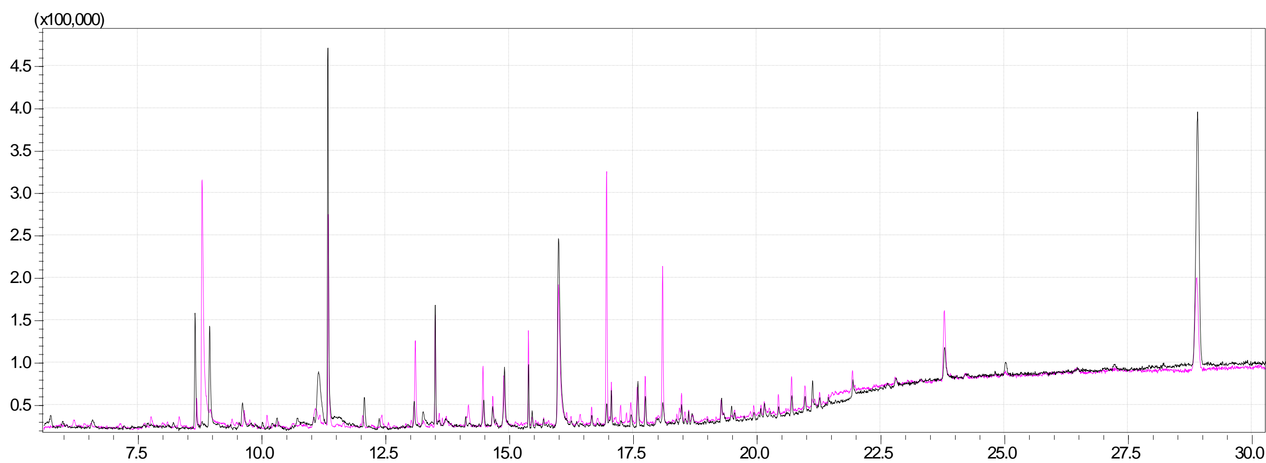

Figura 1. Spontaneous fermentation.

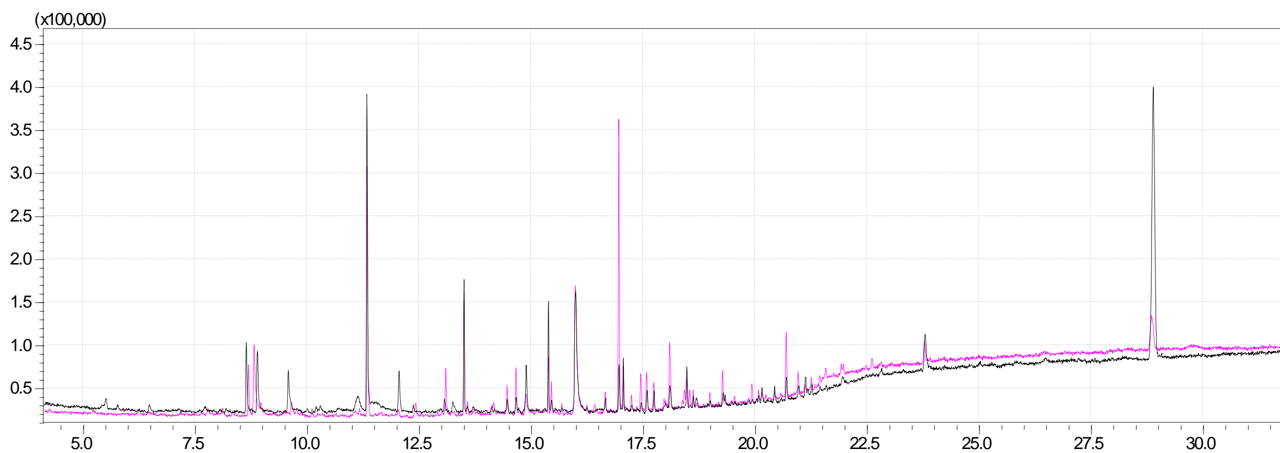

Figura 2.*Bacillus subtilis* CCMA 1234.

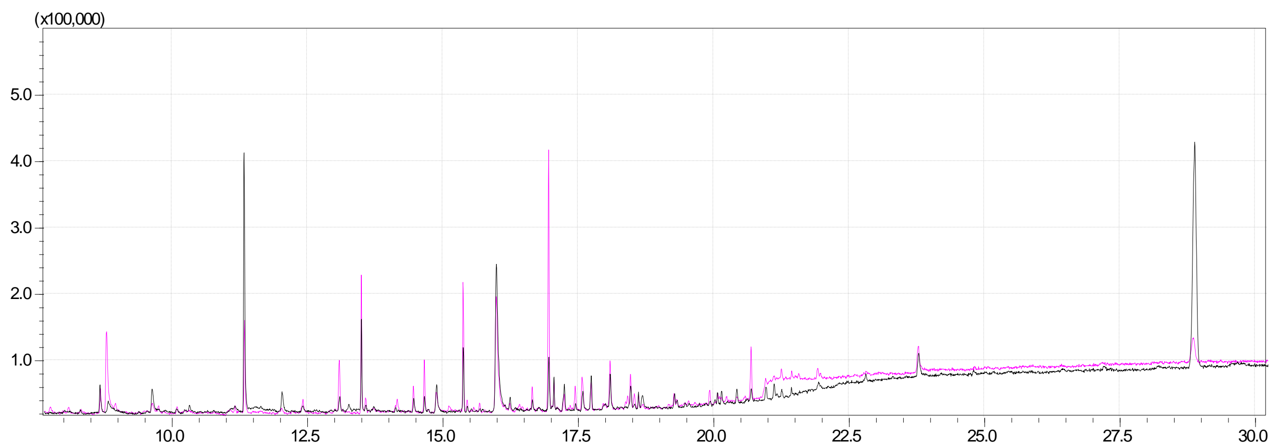

Figura 3.*Bacillus pumilus* CCMA 1251.

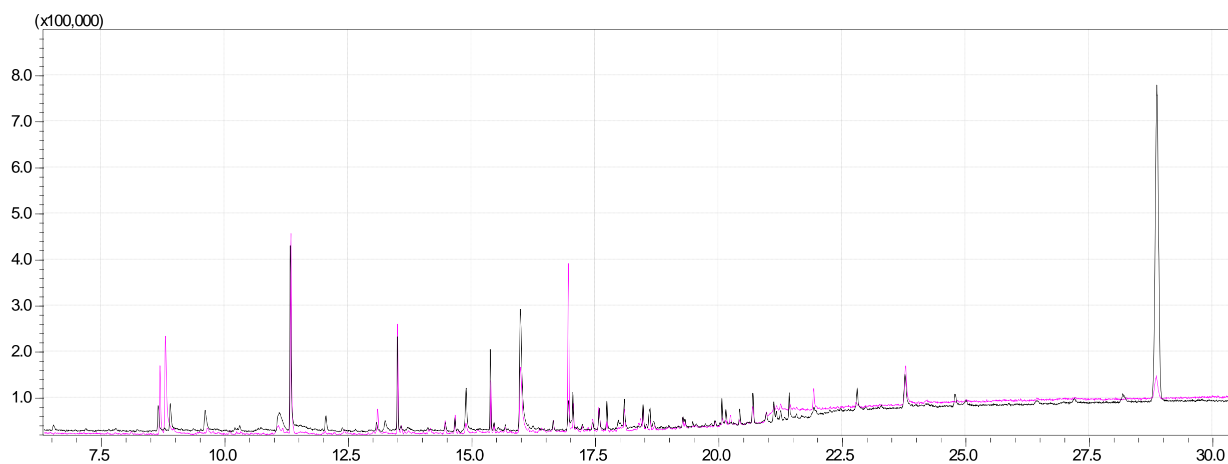

Figura 4.*Cellulosimicrobium cellulans* CCMA1186.

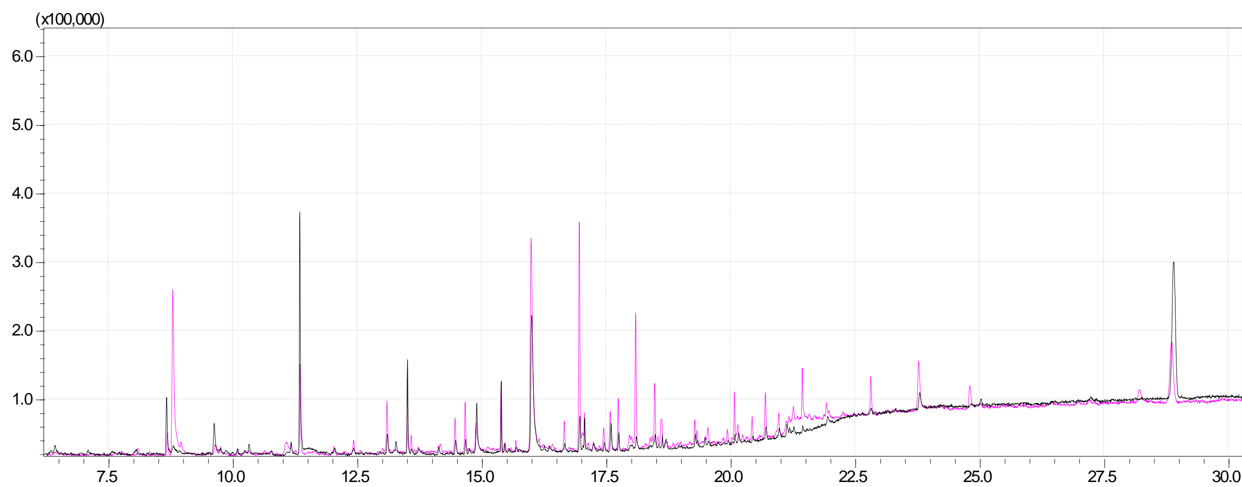

Figura 5. *Pantoea dispersa* CCMA 1203.

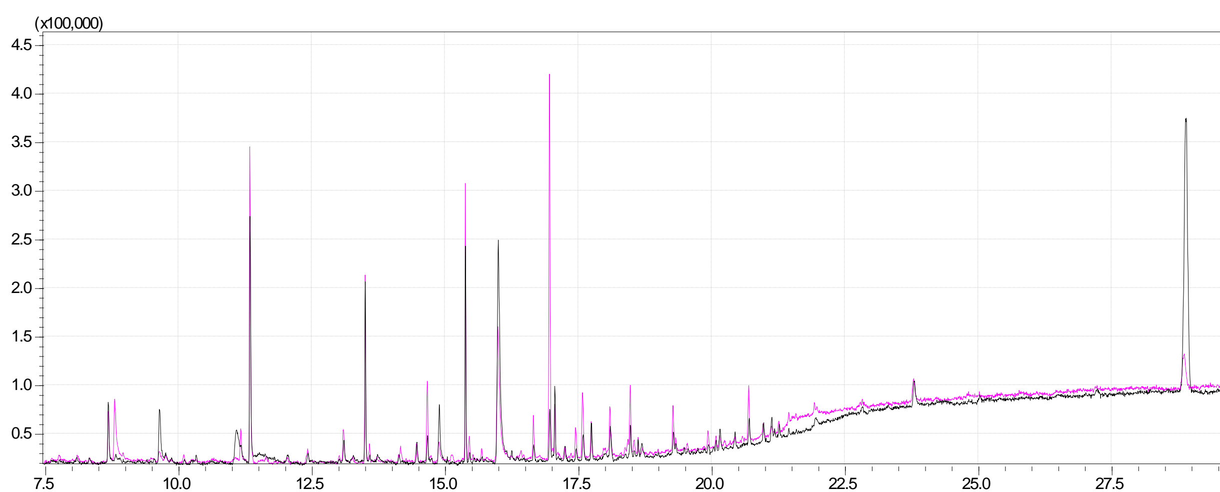

Figura 6. *Microbacterium testaceum* CCMA 1151.

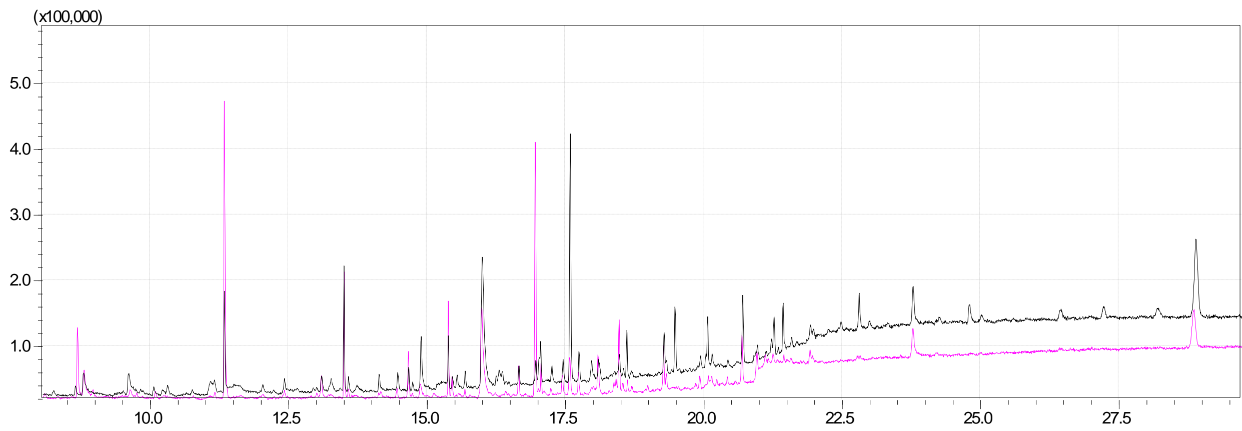

Figura 7.*Leuconostoc mesenteroides* CCMA 1082.

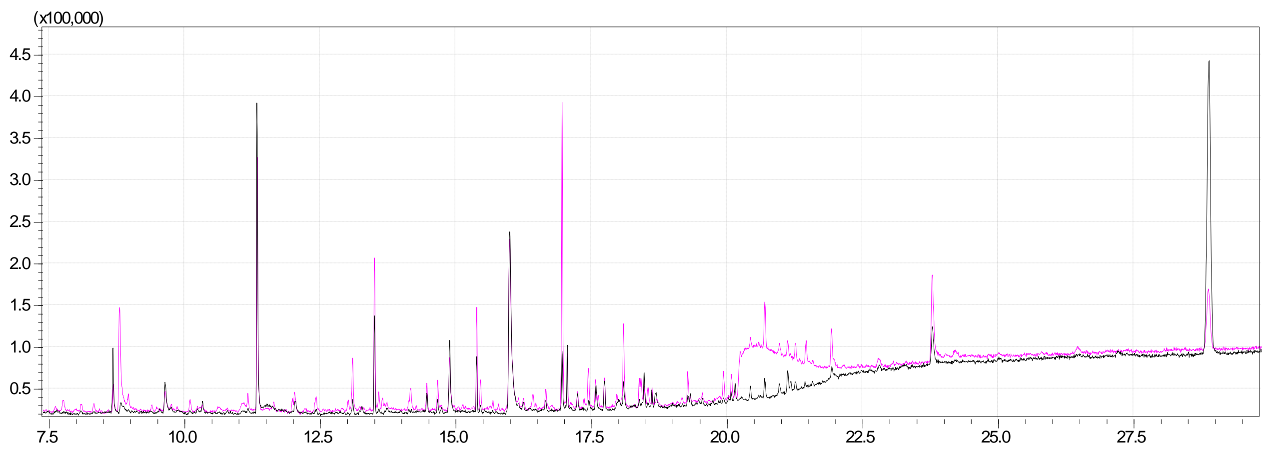

Figura 8.*Leuconostoc mesenteroides* CCMA 1105.

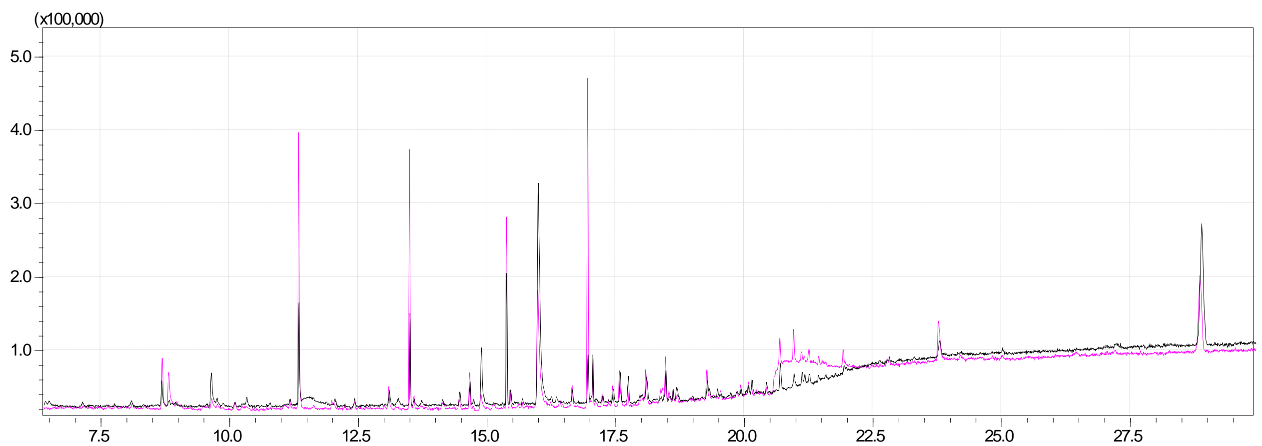

Figura 9.*Lactobacillus plantarum* CCMA 1065.

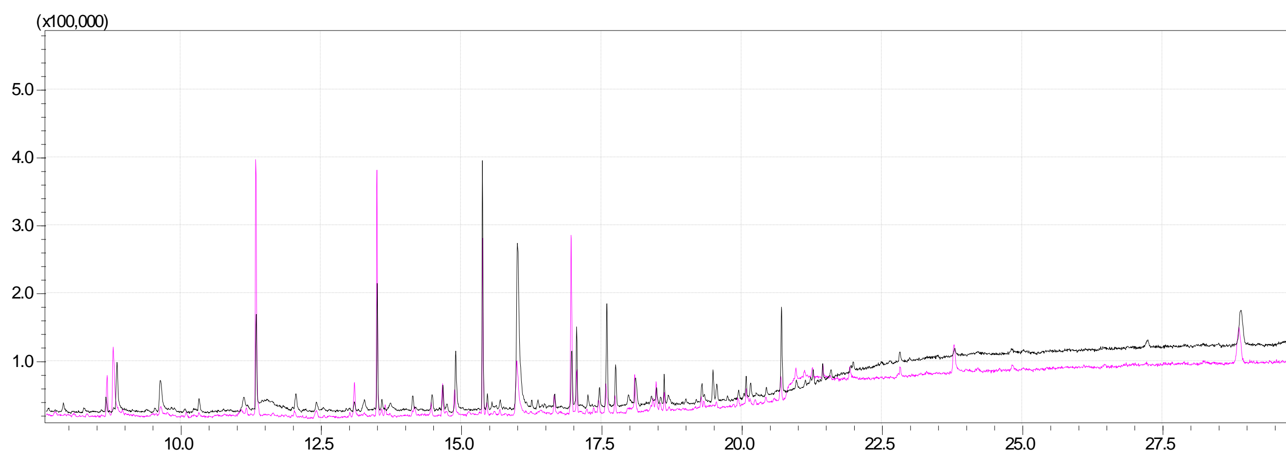

Figura 10.*Lactobacillus plantarum* CCMA 1072.

ANEXO4. Artigo científico resultado da disciplina Pesquisa Orientada publicado no periódico *Food Research International*

Behavior of yeast inoculated during semi-dry coffee fermentation and the effect on chemical and sensorial properties of the final beverage

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Abstract: PulpedMundo Novo and Ouro Amarelo coffee beanswere inoculated with *Saccharomyces cerevisiae* (CCMA 0200 and CCMA 0543) during semi-dry coffee fermentation and compared with a non-inoculated control. Samples were collected throughout the fermentation process (12 days) to evaluate the persistence of the inoculum by Real-Time quantitative PCR (qPCR). Also, the chemical composition of the beans was determined by HPLC and GC–MS and the roasted beans were sensorial evaluated using the cupping test. *S. cerevisiae* CCMA 0543 had an average population of 5.6 log cell/g (Ouro Amarelo cultivar) and 5.5 log cell/g (Mundo Novo cultivar). Citric, malic, succinic and acetic acid were found in all samples, along with sucrose, fructose, and glucose. There were 104 volatile compounds detected: 49 and 55 in green and roasted coffee, respectively. All coffee samples scored over 80 points in the cupping test, indicating they were specialty-grade. Inoculation with the CCMA 0543 strain performed better than the CCMA 0200 strain. This is the first time that qPCR has been used to assess the persistence of the inoculated strains populations during coffee processing. Strain CCMA 0543 was the most suitable as an inoculant due to its enhanced persistence during the process and number of volatile compounds produced.

Keywords: Coffee cupping; Coffee fermentation; qPCR; Saccharomyces cerevisiae; Starter culture

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1. Introduction

Coffee is one of the most widely distributed beverages in the world and ranks among the five most traded agricultural commodities (FAO, 2009). Coffee beans grow inside the fruits of the *Coffea arabica* tree. Upon harvesting, several methods are available to transform the fresh, wet beans to the dried green beans that are traded on the international markets. The choice of this method (wet, dry or semi-dry) influences the chemical composition of the bean as well as the sensory characteristics of the coffee beverage (Silva, 2014).

In the semi-dry method, coffee beans are separated from the fruit by pulping. This step removes the exocarp and most of the mesocarp. The beans are then collected to dry in open air, typically for 10-15 days (depending on weather circumstances). Drying is considered complete when the beans reach 10-12% moisture content. Simultaneous with this drying step, a fermentation process occurs during which, microorganisms degrade remnants of the mesocarp (also called mucilage) that still adhere to the beans (Batista, Chalfoun, Silva, & Schwan, 2016).

In a study of the microbiota associated with the semi-dry process, Vilela, Pereira, Silva, Batista, & Schwan. (2010) observed a microbial succession with bacterial species prevailing in the early stages of fermentation, reaching log 7cfu/g during the first 24 hours of fermentation. Yeasts dominated the later stages (as the moisture content decreased) reaching log 6.9 cfu/g after 5 days. According to Velmourougane, Bhat, Gopinandhan, & Panneerselvam (2011), delays during the semi-dry fermentation and drying process can trigger outgrowth of filamentous fungi, potentially leading to food safety risks and decreased beverage quality. In our lab, Evangelista et al. (2014) inoculated several yeast starter cultures during semi-dry processing of Acaiá cultivar and found that some produced coffee with distinct flavor characteristics in comparison with non-inoculated control samples. However, these data need to be validated through repeat experiments at various farms and harvest seasons, with other varieties and on a commercially relevant scale.

In the present work, two coffee bean varieties (Ouro Amarelo and Mundo Novo) were, respectively, inoculated with two strains of *Saccharomyces cerevisiae*- CCMA 0200 and CCMA 0543 (formerly UFLA YCN727) during semi-dry coffee fermentation. The persistence of the inoculated strains populations was confirmed using Real-Time PCR (qPCR). The effect of the inoculations on the chemical composition of the bean (sugars, acids, and volatiles) was analyzed using gas and liquid chromatography. Finally, a cupping test was performed to evaluate the sensory characteristics of the coffee beverage obtained by these treatments.

2. Materials and Methods

2.1. Microorganisms used as starter cultures

Saccharomyces cerevisiae CCMA 0200 (previously isolated from sugar cane, formed UFLA CA11), and S. cerevisiae CCMA 0543 (formed UFLA YCN 727), were isolated during semi-dry fermentation of coffee beans (Coffea arabica L. var. Acaiá) (Vilela, Pereira, Silva, Batista, & Schwan, 2010) were used as the starter cultures. These strains belong to the Culture Collection of Agricultural Microbiology (CCMA, Federal University of Lavras, Lavras, Minas Gerais, Brazil). *S. cerevisiae* CCMA 0543 already is used as starter culture however, for another coffee variety (Evangelista et al., 2014a; 2014b) and CCMA 0200 is used for another fermentative processes (Ribeiro, Duarte, Dias & Schwan, 2014).

A lyophilized culture of *S. cerevisiae* CCMA 0200 (produced by LNF, Bento Gonçalves, Brazil) was weighed in sterile water at the concentration necessary to inoculate the coffee beans with 5 log cells/g. A culture of *S. cerevisiae* CCMA 0543 stored at -80ºC was reactivated in YEPG tubes containing 9 ml of liquid medium [glucose 20 g/L (Merck, USA), yeast extract 10 g/L (Merck, USA), and bacteriological peptone 10 g/L (Himedia, India), pH 3.5]. The cultures were incubated at 28ºC for 48 h, and then transferred to YEPG (90 ml) and incubated at 28ºC, 150 rpm for 24 h. The yeast cells were transferred to incremental volumes of YEPG until a sufficient number of cells was produced to inoculate the coffee beans with approximately 5 log cells/g. The cells were recovered by centrifugation (7000 rpm, 10 min) and re-suspended in sterile water.

2.2. Harvest and coffee processing

Ouro Amarelo (OA) and Mundo Novo (MN) coffee cultivars were collected mechanically on a farm located in Patrocínio in the state of Minas Gerais, Brazil, at 970–1200 m above sea level, during the harvest of June 2014. Only ripe cherries were used to perform the experiment, since coffee processed with ripe cherries is naturally sweet and have floral and fruit notes while unripe cherries may taste grassy, green, or astringent (Batista et al., 2016). The exocarp and mesocarp of the coffee fruit were separated from the beans in a horizontal pulper (Ecoflex, Pinhalense, São Paulo, Brazil). The pulped coffee beans (60 kg) were spread on suspended terraces. Each treatment was inoculated separately with S. cerevisiae CCMA 0200 and CCMA 0543 and the control samples were not inoculated. The cells suspended in sterile

water were sprinkled on the coffee and mixed manually and aseptically. The fermentations were performed in duplicate. The pulped coffee beans remained on the suspended terraces, where they were regularly turned to ensure uniform drying. The fermentation and drying were considered complete when the beans reached a moisture content of 11–12%. Samples were collected at 0, 24, 48 and 284 h (the final drying time). For each sample, 300 g of beans were aseptically collected in sterile plastic bags and immediately transferred to the Microbial Fermentation Laboratory of the Federal University of Lavras, in iceboxes. Samples were stored at -18°C until microbiological and physicochemical analyses were performed, and at -8 ºC for sensory analyses.

2.3. DNA extraction from pulped coffee and real-time PCR

The total DNA from pulped coffee was extracted using 3 g of sample. Each treatment was mixed with 5ml of ultrapure water for 10 min, then centrifuged at 9000 rpm, 4 ºC for 10 min. The pellet was used for DNA extraction. Total DNA was extracted from samples during the fermentation [at 0, 24, 48 and 284 h (end of drying)] using a QIAamp DNA mini kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions.

Specific primers for the S. cerevisiae were amplified with the primers SC-5fw 5′-AGGAGTGCGGTTCTTTCTAAAG-3′ and SC-3bw 5′-TGAAATGCGAGATTCCCCCA-3′, which span the 26S rDNA region and amplify products 150–200 bp in length (Díaz, Molina, Nähring, & Fischer, 2013). The specificity of primer pair was confirmed by searching in GenBank using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

Real-time PCR was carried out using the Rotor-Gene Q System (Qiagen, Hombrechtikon, ZH, Switzerland). Each reaction comprised 12.5 μl 2× Rotor-Gene SYBR Green PCR Master Mix (Qiagen, Stockach, Konstanz, Germany), 0.8 μM of each primer (Invitrogen, São Paulo, SP, Brazil) and 1 μl template DNA extracted from coffee beans, for a total volume of 25 μl. The mixture was heated to 95ºC for 10 min, followed by 40 cycles of denaturation at 95ºC for 10 s, and annealing/extension at 60ºC for 15 s. The cycling temperature was then increased by 1ºC every 5 s from50ºC to 99ºC to obtain the melting curve. All analyses were performed in triplicate. The DNA concentration in the samples was limited to 50 ng per analysis, except for the standard curves, which were prepared from samples containing a known number of yeast cells. For the standard curves, all yeast species were cultivated in YPD agar at 28ºC for 24 h. The cells were counted using a Neubauer chamber. DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and serially diluted (1:10) from 8 to 3 log cell/mL. Each point on the calibration curve and DNA of samples was measured in triplicate.

2.4. Analysis of acids and sugars by high-performance liquid chromatography (HPLC)

Pulped coffees were ground to a fine powder using an IKA A11 analytical mill. In a 50 ml Erlenmeyer flask, 3 g of powder was mixed with 20 ml of ultrapure water and extracted for 10 min, at room temperature, while stirred with a magnetic bar. The extracts were decanted and centrifuged at 10,000 rpm, 4 C for 10 min. To remove sample components that may precipitate on the chromatographic column, the supernatant was adjusted to pH 2.11 using 200 mM perchloric acid solution before centrifuging a second time under the same conditions. The second supernatant was filtered using a 0.22 μm cellulose acetate filter. The filtered extract was stored at -18 C until analysis.

The extracts were analyzed using an HPLC system (Shimadzu). A Shim-pack SCR-101H (7.9 mm x 30 cm) column was used with a 100 mM solution of perchloric acid and a flow rate of 0.6 ml/min as the mobile phase. The oven temperature was maintained at 50°C for analysis of the acids, detected with a UV detector at 210nm, and at 30°C for analysis of the sugars, detected with a refractive index detector. Analysis was done in triplicate.

2.5. Analysis of volatile compounds by gas chromatography/mass spectrometry (GC/MS)

Volatile compounds were extracted using a manual headspace-solid phase microextraction procedure (HS–SPME) with a divinylbenzene/carboxen/polydimethylsiloxane 50/30 μm SPME fiber (Supelco Co., Bellefonte, PA, USA). Pulped coffee (2 g) was macerated with liquid nitrogen and placed in a 15 mL hermetically sealed vial. After equilibration at 60°C for 10 min, the volatile compounds were extracted at 60°C 30 min. Desorption time on the column was 5 min.

A GCMS-QP2010 (Shimadzu), equipped with an Agilent HP-FFAP column (30 m x 0.25 mm x 0.25 μm) was used for GC/MS analysis. The oven temperature was maintained at 50°C for 5 min, then raised to 190°C at 3°C/min and maintained at 190°C for 10 min. The injector and detector were maintained at 230 and 240°C, respectively. The He carrier gas was maintained at a flow rate of 1.2 ml/min. Analysis was done in duplicate.

2.6. Sensory evaluation

The samples were prepared according to the Specialty Coffee Association of America (Lingle, 2011). The coffee grains were roasted in a laboratory roaster (Probatino, Leogap model, Brazil) with a capacity of 150 g at 180⊚200°C for approximately 10 min, for a light-medium roast and was be used for cupping after 24 hours. The coffee grains were ground in an electric mill (Pinhalense ML-1, Brazil), so that 70-75 percent of the grinds pass through the 20 mesh sieve. For sample uniformity evaluation, 5 cups of each sample were prepared. Cupping vessels used were of tempered glass with capacity of 207 ml and a top diameter of 76 mm. The lids used were made of non-toxic polypropylene. All cups and lid used were of identical volume, dimensions and material of manufacture. Clean and odor-free water was heated approximately 92.2 – 94.4°C and poured directly onto the ground coffee. The ratio was 8.25 g of coffee per 150 mL of water. Cupping spoons used have capacity of 4-5 ml of coffee sample and were made of non-reactive metal. Samples were presented randomly, coded with 3 digits numbers and of monadic way. A panel of three trained coffee experts with Q-Grader Coffee Certificates evaluated the samples for ten attributes, grouped into "subjective" and "objective" categories. "Subjective" attributes were fragrance/aroma, flavor, acidity, body, balance, aftertaste and overall impression. They were scored according to their quality on a scale of 6 to 10 points in intervals of 0.25 points. The "objective" category included uniformity, sweetness, and clean cup (i.e. absence of defects). The objective attributes were scored on a scale from 0 to 10 points, with 2 points awarded for each cup that presented satisfactory levels of each attribute. Besides these attributes, tasters also describe the characteristic flavors of each coffee.

The cupper's preference for the different attributes is evaluated at several different temperatures (2 or 3 times) as the sample cools. Evaluation of the coffee ceased when the sample reaches around 21 ºC and the cupper determined the Overall score. After evaluating the samples, all the scores were added and the Final Score was written in evaluation sheet.
All samples were evaluated in two sessions of two hours each one and in duplicate.

2.7. Statistical analysis

The experiment was carried out in randomized desing. A 2 × 3 factorial arrangement of treatment was used to analyze the results of qPCR, carbohydrates and organic acids and sensory analyses. Two varieties (Ouro Amarelo and Mundo Novo) and three treatments of inoculum (S. cerevisiae CCMA 0200 [Sc0200], S. cerevisiae CCMA 0543 [Sc0543] and without inoculum [control]). The means were compared using a Scott-Knott test. Data were analyzed using software SISVAR (Ferreira, 2014), under model:

$$X = \mu + Varj + Inock + Var \times Inocjk + ejk$$

were, μ = global mean; Varj = varieties effect (j = OA, MN); Inock = inoculum effect (k = Sc0200, Sc0543, control); Var × Inocjk = effect of interaction between varieties and inoculum; ejk = experimental error. Significance was defined at $p<0.05$ level.

3. Results

3.1. Inoculation of yeast in coffee fermentation

The S. cerevisiae populations were quantified in the control and inoculated coffee cultivars during semi-dry fermentation by qPCR (Table 1). According with table 1 there was significant difference between the treatments (CCMA 0200, CCMA 0543 and control – without inoculum) and between varieties. S. cerevisiae CCMA 0543 was present throughout the fermentation (284 h) of both coffee varieties analyzed (Table 1). The population ranged from 4.3◎6.3 log cell/g for coffee variety MN, and 4.8◎5.6 log cell/g for variety OA.

S. cerevisiae CCMA 0200 showed a higher population during the initial fermentation (MN: 4.2 and 4.9 log cell/g and OA: 5.3 and 5.9 log cell/g at 0 and 24 h of fermentation, respectively) compared to the control (MN: 3.4 and 4.4 log cell/g and OA: 3.8 and 3.9 log cell/g at 0 and 24 h of fermentation, respectively), for both coffee varieties. At 48 h, the population of strain CCMA 0543 was higher than CCMA 0200 (Table 1) indicating that CCMA 0543 is more suitable for this process. Already in 284 h fermentation, CCMA 0543 population remained greater than other treatments of both varieties. The population of CCMA 0200 ranged from 3.3◎4.2 log cell/g for MN (Table 1) and from 3.8◎5.9 log cell/g for OA.

The MN and OA coffees inoculated with CCMA 0543 showed a higher population compared to the other treatments tested, increasing the population by 25 (for variety MN) and 74% (for variety OA), at 24 h fermentation. After 48 h fermentation, the population increased 67 (for MN) and 92% (for OA). At 284 h (dried coffee), the increase in population was 30 (for MN) and 33% (for OA). The growth of strain CCMA 0543 was influenced by the coffee variety used (Table 1). The coffee inoculated with CCMA 0200 showed a population increase of 11% (for MN) and 51% (for OA) at 24 h fermentation. After 48 h, the MN variety had a increase (28%) and OA had a population similar to the control. The dried coffee (284 h fermentation) showed no increase in the population for MN and 5% for OA.

3.2. Carbohydrates and organic acids during coffee fermentation

Carbohydrates and organic acids were measured before and after fermentation (Table 2). There was a significant difference between varieties for carbohydrates and organic acids except for malic acid. The treatment CCMA 0543 showed significant difference between other treatments for sucrose, citric, succinic, and acetic acid. The OA coffee inoculated with S. cerevisiae CCMA 0543 showed an increase in the concentration of sugars and organic acids.

Sucrose was the predominant carbohydrate throughout the fermentation (14.6 mg/g at 0 h versus 38.0, 40.0 and 52.1 mg/g at 284 h for the control, CCMA 0200 and CCMA 0543, respectively) in OA beans (Table 2). The MN beans had less sucrose at the start (11.8 mg/g at 0 h) and at end of fermentation (46.6, 34.1 and 40.2 mg/g at 284 h for the control, CCMA 0200 and CCMA 0543, respectively).

Citric, malic, succinic and acetic organic acids were detected and quantified (Table 2). Citric acid was abundant in OA coffee inoculated with S. cerevisiae CCMA 0543 (8.73 mg/g) and was significant different from others treatments, while CCMA 0200 (6.92 mg/g) and in the control sample (5.44 mg/g). The acetic and succinic acid concentrations increased significantly only in the process inoculated with CCMA 0543 (1.86 and 5.78 mg/g, respectively) for OA varietie. Thus, malic acid showed no difference between treatments.

The MN coffee beans showed similar values in all three fermentations analyzed. The most abundant acids were citric and succinic acids. Citric acid was present at 7.52 (control), 6.27 (S. cerevisiae CCMA 0200) and 6.74 mg/g (S. cerevisiae CCMA 0543). Succinic acid was present at 4.91, 5.1 and 4.87 mg/g in the control, S. cerevisiae CCMA 0200 and CCMA 0543, respectively.

Table 1 -Means of main effect and p value of the influence of coffee variety (V) and inoculum (I) and its interactions on *Saccharomyces cerevisiae* populations counting (log cells/g) by qPCR.

	Fermentation time (h)									***p* value**		
	0	**24**	**48**	**284**	**0**	**24**	**48**	**284**	**SEM[b]**	**Variety (V)**	**Inoculum (I)**	**V × I**
Inoculum	**Ouro Amarelo**				**Mundo Novo**							
Control[a]	3.8 aB	3.9 aB	3.7 aB	3.6 aC	3.3 aD	4.4 aA	3.9 aB	3.4 aD	0.17	<0.01	<0.01	<0.01
CCMA 0200	5.3 bB	5.9 bA	5.6 bB	3.8 bE	4.2 bD	4.9 bC	5.0 bC	3.3 aF	0.42			
CCMA 0543	5.6 cD	6.8 cB	7.1 cA	4.8 cE	6.3 cC	5.5 cD	6.2 cC	4.3 bF	0.79			

Data are presented as mean. a-c For each column, mean values with different lowercase letters are significant at $p<0.05$ by Scott–Knott test. A-F For each row, mean values with different capital letters are significant at $p<0.05$ by Scott–Knott test. [a]Control: without inoculant. [b]Standard error of the means.

Table 2 - Effects of inoculation with different strains on concentration of organic acids and carbohydrates (mg\g) present in coffee fermentation via semi-dry processing of Mundo Novo and Ouro Amarelo varieties and *p* value.

	Treatments				*p* value			
Compounds and varieties	**Pulped coffee (0 h)**[a]	**CCMA 0200**	**CCMA 0543**	**Control**[b]	**Variety (V)**	**Inoculum (I)**	**V × I**	**SEM**[c]
Glucose (mg/g)								
Ouro Amarelo	7.7	8.4 aA	9.6 aA	8.9 aA	<0.01	0.76	0.22	0.61
Mundo Novo	7.9	13.2 bA	12.6 bA	12.8 bA				
Fructose (mg/g)								
Ouro Amarelo	11.7	15.0 aA	18.0 aA	15.4 aA	<0.01	0.19	0.21	1.18
Mundo Novo	12.2	23.5 bA	23.6 bA	23.5 bA				
Sucrose (mg/g)								
Ouro Amarelo	14.6	40.0 aA	52.1 bB	38.0 aA	0.15	0.02	0.01	1.91
Mundo Novo	11.8	34.1 aA	40.2 aA	46.6 bA				
Citric acid (mg/g)								
Ouro Amarelo	3.9	6.9 aA	8.7 bB	5.4 aA	0.62	0.05	0.01	0.34
Mundo Novo	3.7	6.3 aA	6.7 aA	7.5 bA				
Malic acid (mg/g)								
Ouro Amarelo	0.6	1.4 aA	1.6 aA	1.3 aA	0.98	0.12	0.07	0.04
Mundo Novo	0.5	1.4 aA	1.5 aA	1.5 aA				
Succinic acid (mg/g)								
Ouro Amarelo	2.6	1.2 aA	5.8 bB	2.4 aA	<0.01	0.01	0.01	0.52
Mundo Novo	3.8	5.1 bA	4.9 aA	4.9 bA				
Acetic acid (mg/g)								
Ouro Amarelo	1.6	1.1 aA	1.9 aB	1.1 aA	<0.01	0.01	0.58	0.13
Mundo Novo	2.3	1.6 bA	2.2 aA	1.8 bA				

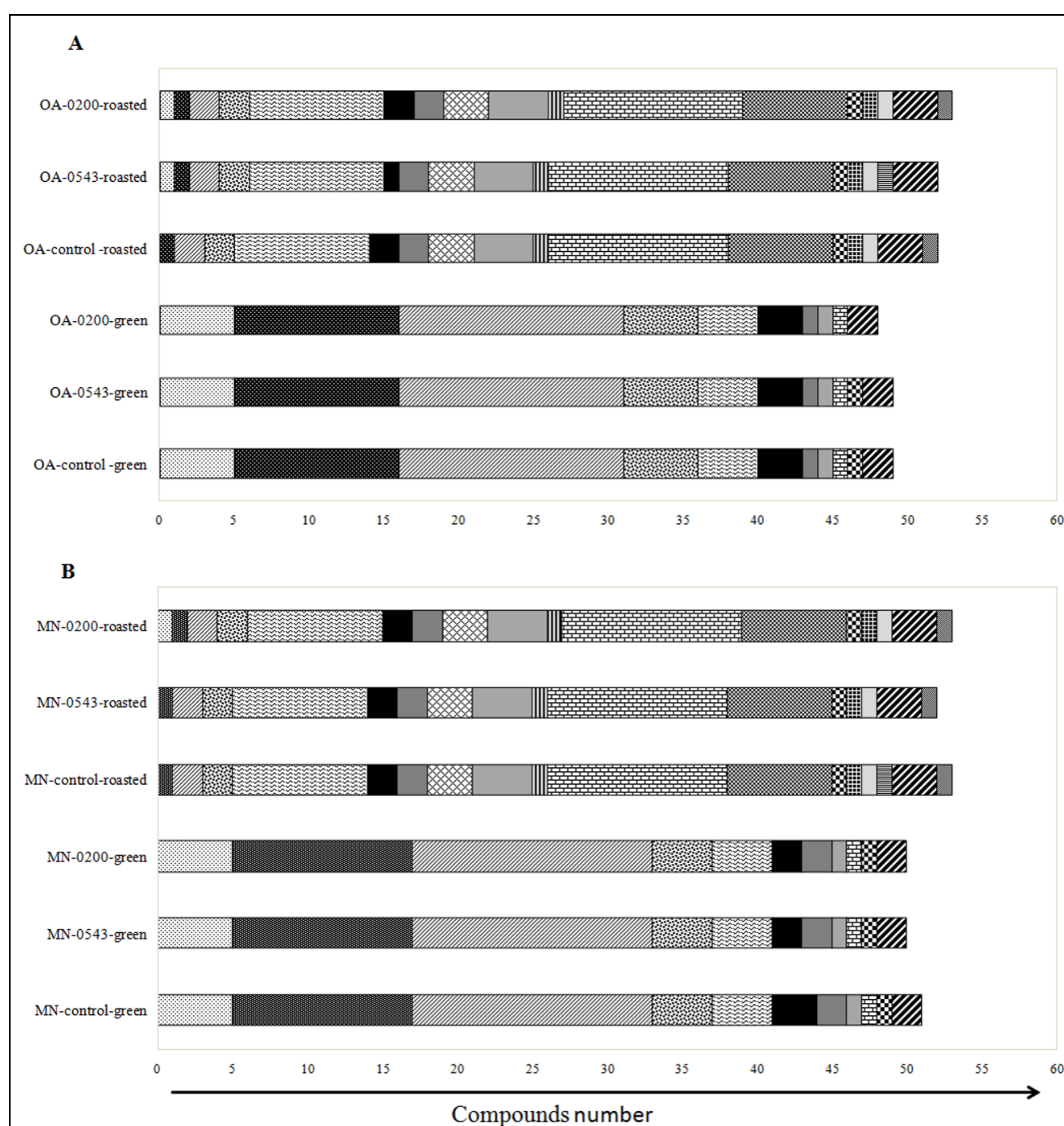
Data are presented as mean. a-b For each column, mean values with different lowercase letters are significant at $p<0.05$ by Scott–Knott test. A-B For each row, mean values with different capital letters are significant at $p<0.05$ by Scott–Knott test. [a]Pulped coffee before fermentation. [b]Control: without inoculum. [c]Standard error of the means

3.3. *GC-MS analyses*

The GC/MS data were used for multivariate analysis, as shown in Figure 1 and Table 4. The figure 1 showed the diference between green and roasted coffee for Ouro Amarelo (Fig 1A) and Mundo Novo (Fig 1B). A total of 96 volatile compounds were detected by HS–SPME/GC/MS (complementary material). Among these compounds, 46 were detected in green coffee and 54 were detected in roasted coffee.

The volatile compounds predominantly found in green coffee belong to acids, alcohols, aldehydes, hydrocarbons, ketones, and furans. Beta-linalool was detected in all treatments, both in green and in roasted coffee, except in OA, inoculated with CCMA 0200, green coffee. This compound belong to terpenes, and produce an important flavor. All classes of compounds that were detected in the green bean were also found in the roasted coffee. In addition to the classes of compounds detected in the green beans was found esters, furaldehydes, pirans, pyrroles, triazoles, thiophenes ans sulphur compounds. The profile of volatile compounds were similar for both varieties.

Figure 1. Classes of volatile compounds identified in green and roasted coffee of Ouro Amarelo (A) and



Mundo Novo (B) varieties. (▒) Acids; (▒) Alcohols; (▨) Aldehydes; (▒) Hydrocarbons; (▒) Ketones; (■) Esters; (■) Phenols; (☒) Furaldehydes; (□) Furans; (▥) Pyrans; (▤) Pyrazines; (▩) Pyrroles; (▣) Terpenes; (▦) Triazoles; (□) Thiophenes; (▤) Sulfur Compounds; (▨) Lactones; (▩) Pyridines.

3.4. *Sensory analysis*

The total scores of the cupping test for coffee variety OA were 81.38, 83.25 and 82.88 for the control fermentation, and those inoculated with CCMA 0200 and CCMA 0543, respectively. The MN coffee had total scores of 84.25 (control), 80.13 (inoculated with CCMA 0200) and 82.63 (inoculated with CCMA 0543). The final score was significant difference between varieties and CCMA 0200 showed difference of the other treatments, since showed the lower score (Table 3).

As shown in table 3, there were no significant differences between the attributes fragrance/aroma and global by the cupping test. The MN control showed slightly more notes than the other treatments. However, the treatment inoculated with S. cerevisiae CCMA 0543 showed better results when compared to the treatment with CCMA 0200. The OA coffee was positively influenced by inoculation with yeasts as starter cultures since inoculation with S. cerevisiae CCMA 0543 and CCMA 0200 had higher scores than the control sample.

Table 3 - Effects of inoculation with different strains on scores of attributes of the cup test, in coffee fermentation via semi-dry processing of Mundo Novo and Ouro Amarelo varieties and *p* value.

Attributes and varieties	Treatments			*p* value			
	Control[a]	CCMA 0200	CCMA 0543	Variety (V)	Inoculum (I)	V × I	SEM[b]
Fragrance/ Aroma							
Ouro Amarelo	7.75 aA	7.63 aA	7.50 aA	0.13	0.13	0.42	0.15
Mundo Novo	7.88 aA	7.63 aA	7.75 aA				
Flavor							
Ouro Amarelo	7.50 aA	7.63 aA	7.63 aA	0.30	0.24	0.08	0.23
Mundo Novo	7.75 aA	7.13 bA	7.50 aA				
Acidity							
Ouro Amarelo	7.00 aA	7.38 aA	7.25 aA	0.77	0.56	0.04	0.27
Mundo Novo	7.63 bA	6.88 aA	7.25 aA				
Body							
Ouro Amarelo	7.38 aA	7.75 aA	7.63 aA	0.72	0.67	0.05	0.20
Mundo Novo	7.75 aA	7.25 bA	7.63 aA				
Aftertaste							
Ouro Amarelo	7.38 aA	7.63 aA	7.75 aA	0.09	0.19	0.01	0.20
Mundo Novo	7.75 bA	7.13 bB	7.38 bB				
Balance							
Ouro Amarelo	7.25 aB	7.63 aA	7.75 aA	0.67	0.02	<0.01	0.32
Mundo Novo	7.75 bA	7.00 bB	7.75 aA				
Overall							
Ouro Amarelo	7.13 aA	7.63 aA	7.38 aA	0.79	0.93	0.06	0.27
Mundo Novo	7.75 aA	7.13 aA	7.38 aA				
Final Score							
Ouro Amarelo	81.38 aA	83.25 aA	82.88 aA	0.78	0.27	0.01	1.24
Mundo Novo	84.25 bA	80.13 bB	82.63 aA				

Data are presented as mean. a-b For each column, mean values with different lowercase letters are significant at $p<0.05$ by Scott–Knott test. A-B For each row, mean values with different capital letters are significant at $p<0.05$ by Scott–Knott test. [a]Control: without inoculum. [b]Standard error of the means.

4. Discussion

In this study, qPCR method was used to detect S. cerevisiae populations during semi-dry coffee fermentation. As shown in Table 1, there was a difference in yeast population between the two coffee cultivars studied. Silva (2014) reported that the microbial activities that occur during fermentation would vary according to the physicochemical properties of the pulp and mucilage and external factors, such as temperature and oxygen availability. The initial population of S. cerevisiae in the control (no inoculation) was 3.3 log cell/g (for MN) and 3.8 log cell/g (for OA). These results were expected because S. cerevisiae has been reported during fermentation of various types of coffee (Silva, 2014).

According to population data found in the present study, the yeast CCMA 0543 showed a better adaptability to coffee fermentation than CCMA 0200 because S. cerevisiae CCMA 0543 was isolated during the coffee processing (Silva, Schwan, Dias, & Wheals, 2000; Vilela et al., 2010), thus, presenting a better physiological adaptation to the process than S. cerevisiae CCMA 0200. The CCMA 0200 yeast has been isolated from a sugarcane fermentation process, being more adapted to alcoholic fermentations. However, Ramos, Dias, Miguel, & Schwan (2014) reported that the inoculation of this strain accelerated the cocoa fermentation process, suggesting that CCMA 0200 might be used in different industrial processes.

The glucose and fructose contents increased from start to end of the process in all treatments analyzed (Table 2). Coffee beans are rich in polysaccharides, such as cellulose and these polysaccharides are hydrolyzed during the fermentation process. The glucose and fructose concentrations are strongly influenced by the processing mode. The levels of these sugars in beans originating from a semi-dry process are between those of wet- and dry-processed beans (Selmar, Bytof, & Knopp, 2008).

As the data showed, the increase in sugar concentrations was influenced by the coffee cultivars. The OA beans presented a higher sucrose concentration when inoculated with S. cerevisiae CCMA 0543 (52.1 mg/g) than the control. This increase in the sucrose concentration may have resulted from the action of enzymes, such as sucrose phosphate synthase, present in the coffee beans. This enzyme is involved in the synthesis of sucrose, which contributes to control the import and mobilization of this carbohydrate (Wendler, Veith, Dancer, Stitt, & Komor, 1990).The presence of the inoculated yeasts influenced the action of this enzyme (due to pH changes) and the sucrose formation process in the grain because the production of acids altered the fermentative environment.

Citric, malic, succinic and acetic acids were detected and quantified in all analyzed samples (Table 2). Some acids increased their concentrations, while others decreased. The same dynamic was observed in both the MN and OA coffee varieties. Similar results were found by Evangelista et al. (2014) using S. cerevisiae UFLA YCN727 as a starter culture in Acaiá coffee. S. cerevisiae CCMA 0200 and CCMA 054, favored the production of desirable acids, such as citric and succinic acid, therefore, they might have positively influenced the final quality of the coffee (Table 2), suggesting their potential use as inoculants in coffee fermentation. It is important to compare the biochemical composition of the bean and cup quality of the various varieties because the variety may alter the final product (Bertrand et al., 2006; Selmar, Bytof, & Knopp, 2008).

The volatile compounds produced during the fermentation and after roasting the coffee beans are presented in Figure 1. According to Batista & Chalfoun (2014), the combination of these techniques is often used to investigate the volatile profiles in defective and non-defective coffee beans.

The aroma and flavor of coffee are attributed to a complex group of chemical constituents that originate from the coffee bean cultivar or variety, fermentation and drying (Bertrand et al., 2006). Among the treatments, MN inoculated with S. cerevisiae CCMA 0543 showed the majority of the 54 volatile compounds associated with several chemical groups. The two factors contributing to the formation of volatile compounds in coffee are microbial metabolites formed during fermentation and the compounds of the inherent grains (Yeretzian, Jordan, Badoud, & Lindinger, 2002). These factors can be very variable depending on the region of coffee production and the coffee bean cultivar, as observed in this study.

A total of 54 volatile compounds were identified after roasting the coffee samples. According to Farah, Monteiro, Calado, Franca, & Trugo (2006), many volatile compounds are detected in roasted and green coffees. After roasting, additional groups of volatile compounds have been identified, such as esters, furaldehydes, furans, ketones, pyrazines, and pyrroles. The presence of furans and ketones were detected in all treatments, which contributed to the citric and herbaceous flavors (Fig. 1). The presence of furans can provide herbal or fruity notes, while ketones are described as providing buttery, caramel-like, musty,

mushroom-like, or fruity notes (López-Galilea, Fournier, Cid, & Guichard, 2006). These groups of volatile compounds were identified in green coffee and roasted grains for all treatments performed in this study (Fig. 1).

In the present study, the cupping test technique was used to evaluate the final product. This technique is used globally for coffee analysis. The choice of the technique for sensory analysis is of extreme importance because there are several techniques of sensory analysis of foods such as Temporal Dominance of Sensations TDS (Evangelista et al., 2014b), Quantitative Descriptive Analysis DQA (Szterk, & Jesionkowska, 2015, Rogalski, Nowak, Fiedor, & Szterk, 2016), check-all-that-apply CATA (Batista, Ramos, Ribeiro, Pinheiro, & Schwan, 2015) among others.

A small difference in the attributes described by the tasters was observed (Table 3). All treatments showed scores higher than 80 points, indicating that the samples are specialty coffees. The OA variety inoculated with yeast presented improvement of attributes notes. Studies have also shown a positive action of yeast as starter cultures in Acaiá coffee (Evangelista et al., 2014). However, the MN-control showed a higher final attributes score than the yeast treatments (inoculation with CCMA 0543 and CCMA 0200), indicating that the inoculation for this variety did not show a striking effect. To better understanding of inoculation effect on the quality of the final product, we indicate the use of other tasting techniques. Despite the worldwide acceptance of the cup taste, particularly when evaluated by trained coffee experts with Q-Grader Coffee Certificates, this is an empirical technique.

5. Conclusions

This study is the first to use qPCR to assess S. cerevisiae population during semi-dry coffee processing. The CCMA 0543 yeast is most suitable for use as coffee inoculants compared to CCMA 0200 yeast. Strain S. cerevisiae CCMA 0543 presented a more favorable biochemical composition of the green coffee and cup quality analysis, than when inoculated in the MN variety. However, the use of starter cultures had a greater effect on the coffee cultivar OA, producing a good quality coffee.

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