SUZANA REIS EVANGELISTA

AVALIAÇÃO DA MICROBIOTA PRESENTE NO PROCESSAMENTO ÚMIDO DO CAFÉ, E DO USO DE CULTURAS INICIADORAS NO PROCESSAMENTO NATURAL E SEMI-SECO

LAVRAS - MG

2014

SUZANA REIS EVANGELISTA

AVALIAÇÃO DA MICROBIOTA PRESENTE NO PROCESSAMENTO ÚMIDO DO CAFÉ, E DO USO DE CULTURAS INICIADORAS NO PROCESSAMENTO NATURAL E SEMI-SECO

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

Orientadora

Dra. Rosane Freitas Schwan

Coorientadoras

Dra. Cristina Ferreira Silva e Batista

Dra. Maria Gabriela da Cruz Pedrozo Miguel

LAVRAS - MG

2014

Ficha Catalográfica Elaborada pela Coordenadoria de Produtos e Serviços da Biblioteca Universitária da UFLA

Evangelista, Suzana Reis.
Avaliação da microbiota presente no processamento úmido do café e do uso de culturas iniciadoras no processamento natural e semi-seco / Suzana Reis Evangelista. – Lavras : UFLA, 2014.
183 p. : il.

Tese (doutorado) – Universidade Federal de Lavras, 2014.
Orientador: Rosane Freitas Schwan.
Bibliografia.

1. Processamento semi-seco. 2. Culturas iniciadoras. 3. Processamento úmido. 4. microbiota. 5. PCR-DGGE. I. Universidade Federal de Lavras. II. Título.

CDD – 663.93

SUZANA REIS EVANGELISTA

AVALIAÇÃO DA MICROBIOTA PRESENTE NO PROCESSAMENTO ÚMIDO DO CAFÉ, E DO USO DE CULTURAS INICIADORAS NO PROCESSAMENTO NATURAL E SEMI-SECO

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

APROVADA em 08 de abril de 2014.

Dr. Disney Ribeiro Dias	UFLA
Dra. Karina Teixeira Magalhães	UFLA
Dra. Cintia Ramos Lacerda	UFLA
Dra. Maísa Honório Belizário	Empresa DATERRA

Dra. Rosane Freitas Schwan
Orientadora

LAVRAS - MG

2014

AGRADECIMENTOS

A Deus que sempre ilumina meu caminho.

A minha orientadora Prof[a]. Dr[a]. Rosane, por todos os ensinamentos, pela confiança, por todas as oportunidades que me proporcionou durante o doutorado.

As minhas coorientadoras professora Cristina e Maria Gabriela pelos direcionamentos, apoio e ensinamentos.

A todos os amigos e colegas do departamento de biologia, do programa de microbiologia agrícola, em especial Fernanda, Alenir, Karla, Angélica e Kely.

A Ivani e Cidinha, por toda ajuda, ensinamento e palavra amiga nos momentos difíceis.

A Cecília, por toda ajuda durante o doutorado, que foi fundamental para o desenvolvimento deste trabalho.

A Rose, pela ajuda e amizade, sempre fazendo por nós além de suas obrigações.

Aos professores e funcionários do Departamento de Biologia e Ciência dos Alimentos da Universidade Federal de Lavras.

Aos membros da banca examidadora: Prof. Dr. Disney Ribeiro Dias, Dr[a]. Karina Teixeira Magalhães, Dr[a]. Cintia Ramos Lacerda e Dr[a]. Maísa Honório Belizário, por participarem da banca examinadora, suas sugestões e correções foram de grande importância para este trabalho.

À Capes, CNPq e FAPEMIG pelo apoio financeiro.

Às fazendas de café Resfriado (Lavras – MG) e a Juliana (Monte Carmelo – MG), por permitirem a realização do trabalho em seu espaço e pelo café.

Aos meus irmãos André e Patrícia, em especial a Pati pela ajuda psicológica em manter a tranquilidade sempre.

Ao meu esposo Felipe, pelo apoio e compreensão, sem você ao meu lado tudo seria mais difícil.

E principalmente aos meus pais, pelo exemplo, apoio, incentivo, confiança e amparo, vocês são minha base e meu exemplo para me manter sempre confiante e nunca querer desistir.

RESUMO GERAL

O café está entre as bebidas mais consumidas, e é um produto de grande importância para a economia do Brasil, principal país produtor e exportador. Existe uma microbiota presente no café composta por leveduras, fungos e bactérias. Durante o processamento dos frutos, a atividade metabólica desta microbiota natural pode influenciar na qualidade final do produto. O presente trabalho teve por objetivo avaliar o uso de leveduras como culturas iniciadoras no processamento natural e semi-seco do café, e conhecer a microbiota presente durante o processamento úmido do café. Métodos dependentes e independentes (PCR-DGGE) decultivo foram utilizados para avaliar a microbiota. As técnicas cromatográficas – cromatografia líquida de alta eficiência (CLAE) e cromatografia gasosa (GC) foram usadas para caracterização do perfil de ácidos e compostos voláteis. A metodologia *Temporal Dominance of Sensations* (TDS) foi utilizada na análise sensorial. O uso de culturas iniciadoras no processamento semi-seco do café, demonstrou ser uma alternativa para se obter um produto diferenciado. Os ácidos propiônico e butírico, que podem prejudicar as características sensoriais, não foram detectados, a presença de culturas iniciadoras promoveu uma maior concentração de voláteis no café torrado e proporcionou um sabor de caramelo, sendo os melhores resultados obtidos para *Candida parapsilosis* UFLA YCN448 e *Saccharomyces cerevisiae* UFLA YCN727. Durante o processamento úmido do café foi possível identificar uma grande variedade de espécies de levedura, sendo as principais *Pichiacaribbica (*53%), *Hanseniaspora uvarum* (35.57%), *Torulaspora delbrueckii* (50%), e de bactérias, sendo as principais *Staphylococcus warneri*, *Erwinia persicina* (19.61%), *Enterobacter asburiae* (41.80 %) e *Leuconostoc mesenteroides*. O uso da técnica de DGGE combinado com o método dependente de cultivo foi necessário para se obter uma melhor avaliação das espécies de micro-organismos presentes durante a fermentação do café.

Palavras-chave: Processamento semi-seco. Culturas iniciadoras. Processamento úmido. Microbiota. PCR-DGGE.

GENERAL ABSTRACT

Coffee is among the most consumed beverages and is a product of great importance for economy in Brazil, the main producer and exporter. There is a microbial flora present in coffee composed by yeast, fungi and bacteria. During the processing of the fruits, the metabolic activity of this natural microbial flora may influence the final quality of the product. The present work aimed at evaluating the use of yeast as initiating culture in the natural and semi-dry processing of coffee, as well as come to know the microbial flora present during the humid processing. Dependent and independent cultivating methods (PCR-DGGE) were used to evaluate the microbial flora. The chromatographic techniques – high performance liquid chromatography (HPLC) and gas chromatography (GC) were used to characterize the profile of volatile acids and compounds. The Temporal Dominance Sensations (TDS) methodology was used in the sensorial analysis. The use of initiating cultures in the semi-dry processing of coffee showed to be an alternative to obtain a differentiated product. The propionic and butyric acids, which may compromise the sensorial characteristics, were not detected, and the presence of initiating cultures promoted a higher concentration of volatile products on toasted coffee and provided a caramel flavor, with the best results being obtained for *Candida parapsilosis* UFLA YCN448 and *Sacchatomyces cerevisiae* UFLA YCN727. During the humid processing of the coffee it was possible to identify a large variety of yeast species, the main species being *Pichia caribbica* (53%), *Hanseniaspora uvarum* (35.57%), *Torulaspora delbrueckii* (50%), and of bacterias, the main species being *Staphylococcus warneri*, *Erwinia persicina* (19.61%), *Enterobacter asburiae* (41.80%) and *Leuconostoc mesenteroides*. The use of the DGGE technique combined with the dependent cultivation method was necessary to obtain a better evaluation of the microorganism species present during coffee fermentation.

Keywords: Semi-dry processing. Initiating cultures. Humid processing. Microbial flora. PCR-DGGE.

SUMÁRIO

PRIMEIRA PARTE

1 INTRODUÇÃO

O café está entre as bebidas mais consumidas mundialmente, e é um produto de grande importância para a economia do Brasil, principal país produtor e exportador. Seu valor no mercado está relacionado diretamente à qualidade. A procura por cafés especiais, como o gourmet, vem aumentando cada vez mais devido ao interesse pelo consumidor por um produto de maior qualidade.

Os frutos do café após serem colhidos, podem ser processados por via natural, semi-seca ou úmida. O tipo de processamento vai interferir diretamente nas características sensoriais do café. A escolha do processamento a ser utilizado depende de vários fatores como espaço para secagem e características climáticas da região. No Brasil, devido ao clima quente e com chuvas escassas na época da colheita do café a via natural é a mais utilizada.

No café, estão naturalmente presentes leveduras, fungos e bactérias. A colonização dos frutos do café por micro-organismos ocorre em todas as etapas de produção desde quando o fruto está na árvore até seu armazenamento. Durante essas etapas, ocorrem mudanças químicas e físicas nos grãos de café selecionando grupos ou espécies microbianas, que através da atividade metabólica podem influenciar na qualidade final do produto (SCHWAN; SILVA; BATISTA, 2012).

É importante o conhecimento da microbiota presente no café e sua influência na qualidade final da bebida, para uma possível formulação e utilização de inóculo na fermentação do café. O uso de inóculo pode trazer diversos benefícios, como redução do tempo de fermentação e consequentemente diminuição do crescimento de micro-organismos

indesejáveis, bem como produção de compostos que incrementem a qualidade sensorial (MASOUD; JESPERSEN, 2006; MASSAWE; LIFA, 2010; SILVA et al., 2013).

Espécies microbianas presentes em frutos de café já foram isoladas e identificadas do processamento pela via natural e seminatural. Alguns gêneros de bactéria encontrados foram *Aeromonas*, *Pseudomonas*, *Enterobacter*, *Serratia*, *Bacillus*, *Klebsiella*, *Arthrobacter*, *Microbacterium*, *Acinetobacte*r e *Escherichia*, e de levedura foram *Pichia*, *Candida*, *Arxula Saccharomycopsis*, *Torulaspora*, *Debaryomyces*e *Rhodotorula* (SILVA et al., 2000, 2008; VILELA et al., 2010).

Neste trabalho, o objetivo foi avaliar a microbiota presente no processamento úmido do café, e o uso de culturas iniciadoras no processamento semi-seco e natural.

2 REFERENCIAL TEÓRICO

2.1 Origem do café

O café provavelmente se originou na província de Kaffa na Etiópia, e foram os holandeses que difundiram o café pela América Central e do Sul, onde hoje seu cultivo comercial tem o maior domínio. O café chegou pela primeira vez à colônia holandesa do Suriname em 1718, sendo posteriormente difundido na Guiana Francesa e então para o Brasil. Em 1730, os britânicos introduziram o café na Jamaica, e por volta de 1825 o café foi plantado no Havaí (SCHWAN; SILVA; BATISTA, 2012).

Pertencente à família botânica Rubiaceae, o café tem cerca de 500 gêneros e mais de 6.000 espécies, mas o gênero Coffea é o mais importante, em termos econômicos (ORGANIZAÇÃO INTERNACIONAL DO CAFÉ- OIC, 2012). Existem mais de 40 espécies conhecidas, mas poucas são usadas na produção do café sendo as mais importantes *Coffea arabica*, *C. canephora*, *C. liberica* e *C. excelsa* (THOMPSON; MILLER; LOPES, 2001), sendo que dessas apenas duas dominam o mercado mundial, o *Coffea arábica* (arábica) e o *C. canephora* (robusta) (SCHWAN; WHEALS, 2003).

O cultivo do café está localizado principalmente nos trópicos, onde não fica exposto ao frio intenso, e em altas altitudes evitando temperaturas muito elevadas (SCHWAN; WHEALS, 2003).

2.2 Importância do café na economia brasileira

O Brasil é o maior produtor e exportador mundial de café, seguido pelo Vietnã, Indonésia e Colômbia, sendo que sua participação na exportação correspondente a 28,08% e do Vietnã, segundo maior exportador 22,81%

segundo dados de 2013 (ASSOCIAÇÃO BRASILEIRA DA INDÚSTRIA DE CAFÉ- ABIC, 2013). O café está entre os cincos principais produtos exportados pelo Brasil, sendo comercializado principalmente como café em grão seguido pelo tipo solúvel (BRASIL, 2011). Os cafés especiais no Brasil, como descafeinado, gourmet, sustentáveis e certificados, embora representem a menor parte do mercado, vem apresentando desde alguns anos, um aumento no consumo, e novas marcas de cafés especiais de alta qualidade estão sendo lançadas no mercado brasileiro (ABIC, 2010).

A produção de café, na safra 2014 no Brasil, está estimada em 50,15 milhões de sacas beneficiadas, e o café arábica está com uma produção estimada em 37,53 milhões de sacas (75,1% da produção nacional), sendo que o estado de Minas Gerais é o maior produtor (COMPANHIA NACIONAL DE ABASTECIMENTO- CONAB, 2014).

2.3 Composição do café

O fruto do café é constituído normalmente por duas sementes envolvidas por uma fina membrana conhecida como película prateada, que são protegidas por uma camada rígida chamada pergaminho, no qual está aderido firmemente o mesocarpo mucilaginoso (mucilagem), seguido pela polpa que é coberta pela casca (exorcarpo), que protege o fruto (Figura 1) (ARUNGA, 1982; SCHWAN; WHEALS, 2003).

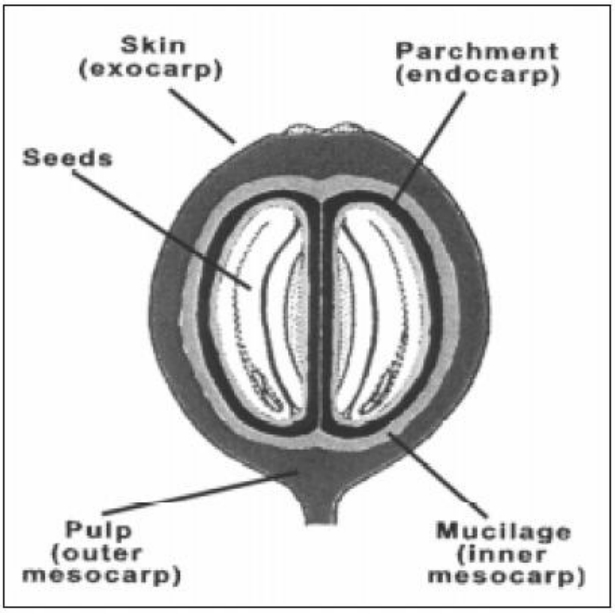

Figura 1 Sessão longitudinal do fruto do café
Fonte: Avallone et al. (2000)

A polpa é composta basicamente por 76% de água, 10% de proteína, 2% de fibra, 8% de sais minerais e 4% de diferentes compostos solúveis e insolúveis como pectina, taninos, açúcares redutores e não redutores, cafeína, ácidos clorogênicos e cafeicos, celulose, hemicelulose, lignina e aminoácidos (ELÍAS, 1978).

A camada de mucilagem está localizada entre a polpa e o pergaminho, e representa 5% do peso seco do fruto (SCHWAN; WHEALS, 2003). Apresenta em sua composição água, açúcares, substâncias pécticas, ácidos orgânicos (ELÍAS, 1978), holocelulose, lipídeos e proteínas. Dentre esses componentes um dos mais importantes são as substâncias pécticas (ARUNGA, 1982), que correspondem a 30% dos polissacarídeos constituintes da mucilagem (AVALLONE et al., 2000).

As substâncias pécticas são compostas por cadeias de ácido galacturônico com unidades parcialmente metiladas com grupos carboxílicos, e grupos hidroxi acetilados, sendo classificadas, de acordo com as variações nessa estrutura, em protopectina, ácidos pectínicos, pectina e acidos pécticos (ARUNGA, 1982). A pectina contida na mucilagem e polpa contém cerca de 60% de ácido urônico, com elevado grau de metilação (em torno de 62%), e grau moderado de acetilação (cerca de 5%) (AVALLONE et al., 2000).

2.4 Processamento do café

A colheita do café pode constituir-se de frutos verdes, maduros (cereja), secos (boia), folhas, ramos, terra, paus e pedra, sendo a proporção de cada um destes constituintes dependentes do sistema e cuidados com a colheita. Para separação do café, é realizada a abanação, que irá retirar as folhas e outras impurezas mais leves e em seguida o café é lavado, separando os grãos de acordo com a densidade. Os mais densos são os verdes e cerejas, e o de menor densidade o café boia. Essas duas porções deverão ser secas separadamente, formando lotes mais homogêneos, permitindo assim secagem uniforme (BORÉM, 2004).

O processamento do café pode ser realizado de diferentes formas, dentre elas estão à via natural, semi-seca e úmida. A forma com que o café é processado influencia na sua qualidade final, devido à variação de compostos como ácido clorogênico, trigonelina e açúcares (DUARTE; PEREIRA; FARAH, 2010; FARAH et al., 2006; KNOPP; BYTOF; SELMAR, 2005). Os compostos voláteis também podem variar de acordo com o tipo de processamento (GONZALEZ-RIOS et al., 2007).

2.4.1 Via Natural

A via natural é o modo mais antigo e mais simples de processar o café recém-colhido. No Brasil e na Etiópia, é o método mais utilizado, e consiste em submetê-lo à secagem na sua forma integral, durante um período que varia de 10 a 25 dias, quando ocorre a fermentação microbiana natural, e enzimas são liberadas modificando a polpa e mucilagem, podendo influenciar na qualidade do café (SCHWAN; WHEALS, 2003; SILVA et al., 2000). Esse processo é que menos afeta a condição natural do café, pois todas as suas estruturas são mantidas (BORÉM, 2004).

O exocarpo, originalmente vermelho ou amarelo, torna-se escuro durante a secagem, formando o conhecido café coco. O processamento natural é muito utilizado nas regiões tropicais, onde há uma estação seca característica, no período de colheita (BORÉM, 2004).

2.4.2 Via semiseca

O processamento semi-seco (semi-seco, natural despolpado) é um intermediário entre o natural e o úmido. Nesse processo o café deixa de ser natural por ter estruturas removidas. O café tem o exocarpo (casca) e o mesocarpo (mucilagem) removidos, ou somente a casca, e então levado pra secagem em terreiros (VILELA et al., 2010).

Neste tipo de processamento, necessita-se de menor tempo de secagem, e possui menor chance de ocorrer fermentações indesejáveis. As sementes obtidas desse processamento são chamadas de café natural despolpado, e são muito apreciadas em misturas para café expresso (TEIXEIRA et al., 1995).

2.4.3 Via úmida

O processamento pela via úmida é comum na Colômbia, América Central e no Havaí, o café, após passar pelo descascamento, é levado para tanques com água por 24 a 36h onde ocorre a fermentação por micro-organismos naturalmente presentes no grão que secretam pectinases degradando a mucilagem péctica tornando-a facilmente removível pela água (SCHWAN; WHEALS, 2003; SILVA et al., 2000). A fermentação ocorre em tanque de concreto ou madeira, por um período que pode variar entre 12 a 60 horas, dependendo das condições ambientais, maturação dos frutos e variedade processada (THOMPSON; MILLER; LOPES, 2001), sendo o tempo ideal de 24 a 48 horas (SCHWAN; WHEALS, 2003). Deve-se ter um cuidado especial nesta etapa para que não ocorram fermentações indesejáveis. Posteriormente os grãos são lavados, para eliminar os resíduos e conduzidos à secagem (BORÉM, 2004).

2.5 Substâncias e enzimas pécticas

As substâncias pécticas são coloidais e constituídas, na sua maioria, por cadeias de ácidos D-galacturônicos ligados em α (1 → 4), com grupos carboxílicos que podem estar parcialmente metoxilados e parcial ou totalmente neutralizados por bases (Figura 2), podendo ser classificadas em protopectinas, ácidos pectínicos, ácidos pécticos e pectina (ARUNGA, 1982).

A protopectina é insolúvel em água e é constituída de completa esterificação, está ligada a outros constituintes das células vegetais (ARUNGA, 1982). Ácidos pécticos são cadeias de ácidos D-galacturônicos, livres de metoxilas. Ácidos pectínicos, são constituídos por ácidos poligalacturônicos com número significativo de metoxilas na forma de ésteres e não são necessariamente solúveis em água. Pectinas são solúveis em água, com número de metoxilas

esterificadas e grau de neutralização variável, e algumas pedem ter grupos hidroxílicos acetilados (JAYANI; SAXENA; GUPTA, 2005).

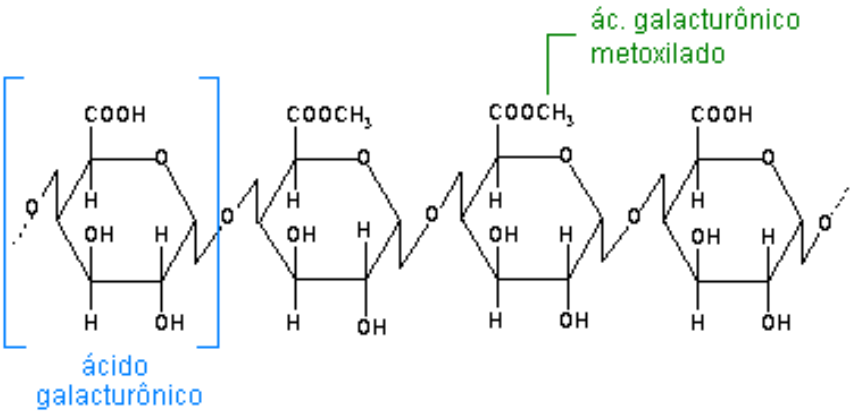

Figura 2 Estrutura molecular da pectina
Fonte: Ecured (2014)

As enzimas pectinolíticas podem ser dividias em três grupos: protopectinases, que degradam a protopectina insolúvel dando origem à pectina polimerizada altamente solúvel; as esterases (desesterificante ou desmetoxilante) removem os grupos metil éster; e as despolimerizantes (incluem as enzimas hidrolíticas e as liases) catalisam a clivagem hidrolítica das ligações α (1 → 4) glicosídicas das substâncias pécticas. Dependendo da ação das enzimas, se ela é aleatória ou terminal, estas são denominadas como endo ou exo enzimas respectivamente (JAYANI; SAXENA; GUPTA, 2005; KASHYAP et al., 2001; UENOJO; PASTORE, 2007).

As protopectinases, que também podem ser chamadas de pectinosinases catalisam a reação da protopectina insolúvel com moléculas de água dando origem à pectina solúvel. As poligalacturonases (PG) são enzimas pectinolíticas que catalisam a clivagem hidrolítica da cadeia de ácido poligalacturônico com a

introdução de água através da ponte de hidrogênio (JAYANI; SAXENA; GUPTA, 2005; KASHYAP et al., 2001; UENOJO; PASTORE, 2007).

As liases, também chamadas transeliminases, realizam a quebra não hidrolítica, rompem ligações glicosídicas resultando em galacturonídeos com ligação insaturada entre os carbonos 4 e 5 do ácido galacturônico formado, e incluem a pectina liase (polimetilgalacturnato liase, PMGL) e pectato liase (poligalacturonato liase, PGL). A pectina esterase (PE), (pectinametilesterase) catalisa a desesterificação de ligações ésteres metílicos da pectina, liberando pectinas ácidas e metanol. A pectina resultante sofre a ação de poligalacturonases e liase (JAYANI; SAXENA; GUPTA, 2005; KASHYAP et al., 2001; UENOJO; PASTORE, 2007).

2.6 Microbiota e fermentação do café

A fermentação do café ocorre pela degradação da mucilagem ao redor do grão, que apresenta grandes quantidades de substancia péctica (AGATE; BHAT, 1966). A ação microbiana na polpa mucilaginosa resulta na produção de álcoois e ácidos no qual se difundem para a semente ocasionando reações bioquímicas que interferem na qualidade final do produto (SCHWAN; WHEALS, 2003).

Estudos vêm sendo realizados há muitos anos para avaliar a presença de micro-organismos no café e sua relação com a degradação da mucilagem. Frank, Lum e Cruz (1965) avaliaram em Kona, distrito do Havaí, a população microbiana presente no café despolpado, durante a fermentação em frascos, verificando a presença predominante de espécies de bactérias gram-negativas e fermentadoras de lactose. Dentre as culturas isoladas estão as espécies *Erwinia*, *Paracolobactrum* e *Escherichia*. Os isolados foram testados quanto à capacidade

de degradar a mucilagem, e as bactérias *Erwinia dissolvens* apresentou essa característica.

Pee e Castelein (1971) identificaram em café robusta leveduras *C. guilliermondii* na superfície e mucilagem dos grãos; *C. parapsilopsis, S. cerevisiae, Torulopsis famata, S. marxianus, C. tropicalis, Rhodotorula mucilaginosa* e *C. pelliculosa* na superfície dos grãos.

Avallone et al. (2001) avaliaram no México a microbiota presente em café processado pela via úmida, observando a presença principalmente de bactérias Gram-negativas sendo os gêneros mais frequentes *Erwinia* e *Klebsiella*. Durante a fermentação, houve um aumento de bactérias acido lácticas e leveduras, esses autores relacionaram a fermentação da polpa com a utilização de açúcares simples, pois no meio sintético de pectina, foram isolados somente bactérias incapazes de utilizar a pectina presente no café.

As leveduras estão presentes no café durante o processo de fermentação e secagem. Masoud et al. (2004) isolaram diferentes espécies presentes em café arábica na Tanzânia (África) processados por via úmida, em diferentes estágios da fermentação e durante a secagem. *Pichia kluyveri* e *Pichia anomala* foram encontradas em maior número durante a secagem. *Hanseniaspora uvarum* predominou durante a fermentação, porém diminuiu durante a secagem. Outras espécies encontradas foram: *Kluyveromyces marxianus, Candida pseudointermedia, Issatchenkia orientalis, Pichia ohmeri, Torulaspora delbrueckii, Saccharomyces cerevisiae* e *Candida xestobii*.

A microbiota presente no café pode variar de acordo com a região de cultivo, variedade do café e processamento utilizado, entre outros fatores (SILVA et al., 2000). Agate e Bhat (1966) detectaram a presença das espécies de leveduras *S. marxianus, S. bayanus, S. cerevisiae* var. *ellipsoideus*, e *Schizosaccharomyces sp*. e dos seguintes gêneros de bactérias, *Streptococcus*, *Pseudomonas, Flavobacterium*, e *Proteus*, em café robusta da Índia, e a

incidência de leveduras foi maior que a de bactérias. Devido à presença de um grande número de leveduras pectinolíticas no café, estas podem ter um importante papel na degradação da mucilagem.

No Brasil, alguns estudos foram realizados em relação à microbiota do café e o processo de fermentação. Em relação ao café processado pela via natural, Silva et al. (2000) avaliaram amostras de café arábica de diferentes fazendas do estado de Minas Gerais, durante a fermentação e em diferentes estágios de maturação, (cereja, passa e seco) em dois anos. Devido ao café natural possuir maior quantidade de substratos, foi encontrada grande diversidade de micro-organismos. As bactérias foram encontradas em maior abundância, e as leveduras apresentaram aumento durante o processo de fermentação. Os gêneros mais incidentes para bactérias foram *Aeromonas, Enterobacter, Pseudomonas, Serratia, Lactobacillus* e *Bacillus*, e em relação às leveduras foram *Pichia, Cândida* e *Arxula,* com tendência a aparecerem em frutos secos e fermentados. Os fungos identificados ao longo da secagem e estocagem do café foram dos gêneros *Cladosporium, Fusarium* e *Penicillium* com maior incidência na estocagem e estavam presentes em todas as fazendas.

Durante a secagem do café natural em terreiro, ocorre uma fermentação espontânea devido à presença de diversos micro-organismos. Inicialmente a população de bactéria é predominante, representando 93% do total dos micro-organismos isolados, sendo as leveduras e fungos pouco encontrados. No decorrer dos dias com a diminuição da umidade e atividade de água, há uma sucessão microbiana ocorrendo e um aumento na população de leveduras e fungos e diminuição de bactérias (SILVA et al.,2008).

Em relação aos isolados obtidos no processamento natural do café, as bactérias gram-positivas representam a maior parte, sendo o gênero *Bacillus* predominante, bactérias gram-negativas também são encontradas e os principais gêneros são *Serratia, Enterobacter* e *Acinetobacter*. Dentre as leveduras, os

gêneros que se destacam são *Debaryomyces, Pichia* e *Cândida*, e para os fungos *Aspergillus*, *Penicillium, Fusarium* e *Cladosporium* (SILVA et al.,2008).

Vilela et al. (2010) avaliaram a microbiota presente em café arábica cereja, durante o processamento seminatural (semi-seca), onde o café é mecanicamente despolpado e então colocado em terreiro para secagem. Semelhante ao processamento natural a população de bactérias predominou. Porém em relação aos fungos, após 120 horas de fermentação não foram mais detectados. *Bacillus subtilis, Escherichia coli, Enterobacter agglomerans, Bacillus cereus* e *Klebsiella pneumoniae,* foram as espécies mais encontradas de bactérias. Em relação às leveduras, as espécies que se destacaram foram *Pichia anomala, Torulaspora delbrueckii* e *Rhodotorula mucilaginosa.* Dentre os fungos encontrados no início da fermentação, *Aspergillus* foi o gênero predominante.

2.7 Uso de culturas iniciadoras e produção de enzimas

O uso de culturas iniciadoras melhora a qualidade de alimentos fermentados, trás benefícios gerais à fermentação, como um melhor controle do processo, e previsibilidade dos produtos. Essas culturas são selecionadas como cepas simples ou múltiplas (HOLZAPFEL, 2002).

No processamento do café o uso de inóculo selecionado durante a fermentação, composto por uma microbiota adequada, pode ser útil, devido à remoção mais rápida da mucilagem e liberação de compostos que melhoram o flavor do café. Este procedimento não necessita de muito custo adicional, sendo vantajoso para o produtor. A adição de enzimas pectinolíticas sobre o café no processamento natural acelera o processo, porém é um tratamento caro, sendo importante o estudo e o isolamento de leveduras pectinolíticas que secretam essas enzimas em quantidades adequadas, para que possam ser adicionadas por

aspersão acelerando o processo e trazendo outros benefícios (SCHWAN; WHEALS, 2003).

Muitas pectinases comerciais estão disponíveis comercialmente e podem ser utilizadas na fermentação do café. Estas enzimas, que são geralmente uma mistura de enzimas pécticas, podem conter também hemicelulases e celulases, porém devido a restrições financeiras, não têm sido amplamente utilizadas (ARUNGA, 1982). Nos dias de maior colheita, os custos adicionais do uso de enzimas podem ser viáveis devido à redução no tempo de remoção da mucilagem (PUERTA-QUINTERO, 2009).

Dentre os micro-organismos presentes no café, alguns podem produzir enzimas, que aceleram a degradação da mucilagem e, consequentemente, o processo de fermentação, como as leveduras. Algumas espécies de leveduras como *D. hansenii* e *P.guilliermondii* são capazes de crescerem em meio sintético de pectina, comprovando a importância desses micro-organismos na degradação da mucilagem do café (SILVA et al., 2008).

Masoud e Jespersen (2006) avaliaram a capacidade de produzir pectinases, das leveduras *P. anomala*, *P. kluyveri* e *H. uvarum* isoladas do café arábica processado através da via úmida. Poligalacturonase foi produzida em grande quantidade pelas espécies *P. anomala* e *P. kluyveri*, podendo estar envolvidas na degradação da pectina durante a fermentação do café.

Sakiyama et al. (2001) isolaram bactérias endofíticas de grão de café, e identificaram *Paenibacillus amylolyticus*, como produtora de pectina liase, alguns fungos já tinham sido isolados e identificados como produtores dessa enzima, mas raramente uma bactéria.

A adição de culturas iniciadoras pode trazer outros benefícios, como à inibição do crescimento de micro-organismos indesejáveis, dentre eles o fungo *Aspergillus. ochraceus*, produtor de ocratoxina A, que pode ter seu crescimento inibido pelo uso das leveduras *P. anomala*, *P. kluyveri* em conjunto com

bactérias ácido lácticas (MASSAWE; LIFA, 2010). O mesmo foi observado pelos autores Masoud e Kaltoft (2006), que verificaram que além da *Pichia anomala* e *Pichia kluyveri*, que são micro-organismos predominantes na fermentação do café, *Hanseniaspora uvarum* também pode inibir o crescimento de *Aspergillus ochraceus*.

Djossou et al. (2011) investigaram a capacidade de bactérias ácido lácteas em inibir o crescimento do fungo *Aspergillus carbonarius*, ambos isolados do café, observando que o grupo de bactérias *L. plantarum sp* apresentou potencial antifúngico no crescimento de *A. carbonarius.*

Em estudo realizado por Silva et al. (2013), as leveduras das espécies *S. cerevisiae* UFLA CN727, *P. guilliermondii* UFLA CN731 e *C. parapsilosis* UFLA CN448, isoladas do processamento natural e semi-seco do café, apresentam bom potencial para serem usadas como culturas iniciadoras. Estes isolados foram selecionados entre outros por produzirem enzimas que podem agir na mucilagem acelerando e melhorando o processo de fermentação, e por produzirem ácidos orgânicos e compostos voláteis que contribuem para qualidade final da bebida.

REFERÊNCIAS

AGATE, A. D.; BHAT, J. V. Role of pectinolytic yeasts in the degradation of mucilage layer of *Coffea robusta* cherries. **Applied Microbiology**, New York, v. 14, n.2, p. 256-260, 1966.

ARUNGA, R. O. Coffee. In: ROSE, A. H. (Ed.). **Economic microbiology:** fermented foods. Amsterdam: Elsevier, 1982. p. 259-279.

ASSOCIAÇÃO BRASILEIRA DA INDÚSTRIA DE CAFÉ. **Exportação mundial do café**. São Paulo, 2013. Disponível em: <http://www.abic.com.br/publique/cgi/cgilua.exe/sys/start.htm?sid=49>.Acesso em: 9 mar. 2014.

ASSOCIAÇÃO BRASILEIRA DA INDÚSTRIA DE CAFÉ. **Pesquisa:** tendência de consumo de café. São Paulo, 2010. Disponível em:<http://www.abic.com.br/publique/cgi/cgilua.exe/sys/start.htm?sid=45>. Acesso em: 9 mar. 2014.

AVALLONE, S. et al. Microbiological and biochemical study of coffee fermentation. **Strain**, London, v. 42, n. 4, p. 252-256, Apr. 2001.

AVALLONE, S. et al. Polysaccharide constituents of coffee-bean mucilage. **Journal of Food Science**, Chicago, v. 65, n. 8, p. 1308-1311, 2000.

BORÉM, F. M. **Pós-colheita do café**. Lavras: UFLA, 2004. 127 p.

BRASIL. Ministério da Agricultura, Pecuária e Abastecimento. **Informe estatístico do café**.Brasília,2011. Disponível em: <http://www.agricultura.gov.br/acesso-a-informacao/estatistica. Acesso em: 10 mar. 2014.

COMPANHIA NACIONAL DE ABASTECIMENTO. **Acompanhamento da safra brasileira:** primeira estimativa da safra de 2014. Disponível em: <http://www.conab.gov.br/>. Acesso em: 3 mar. 2014.

DJOSSOU, O.et al. Robusta coffee beans post-harvest microflora: *Lactobacillus plantarum* sp. as potential antagonist of *Aspergillus carbonarius*. **Anaerobe**, London, v. 17, n. 6, p. 267-272, Dec. 2011.

DUARTE, G. S.; PEREIRA, A. A.; FARAH, A. Chlorogenic acids and other relevant compounds in Brazilian coffees processed by semi-dry and wet post-harvesting methods. **Food Chemistry**, London, v. 118, n. 3, p. 851-855, Feb. 2010.

ECURED. **Pectina**. Disponível em: <http://www.ecured.cu/index.php/Pectina>. Acesso em: 10 fev. 2014.

ELÍAS, L. G. Composición quimica de la pulpa de café y otros subproductos. In: BRAHAM, J. E.; BRESSANI, R. (Ed.).**Pulpa de café:** composición, tecnología y utilización. Panamá: INCAP, 1978. p. 19-29.

FARAH, A. et al. Correlation between cup quality and chemical attributes of Brazilian coffee. **Food Chemistry**, London, v. 98, n. 2, p. 373-380, Jan.2006.

FRANK, H. A.; LUM, N. A.; CRUZ, A. S. D. Bacteria responsible for mucilage-layer decomposition in kona coffee cherries. **Applied Microbiology**, New York, v. 13, n. 2, p. 201-207, 1965.

GONZALEZ-RIOS, O.et al. Impact of "ecological" post-harvest processing on the volatile fraction of coffee beans: I. green coffee. **Journal of Food Composition and Analysis**, San Diego, v. 20, n. 3/4, p. 289-296, May 2007.

HOLZAPFEL, W. H. Appropriate starter culture technologies for small-scale fermentation in developing countries. **International Journal of Food Microbiology**, Amsterdam, v. 75, n. 3, p. 197-212, May 2002.

JAYANI, R. S.; SAXENA, S.; GUPTA, R. Microbial pectinolytic enzymes: a review. **Process Biochemistry**, London, v. 40, n. 9, p. 2931-2944, Sept. 2005.

KASHYAP, D.R. et al. Applications of pectinases in the commercial sector: a review. **Bioresource Technology**, Essex, v. 77, n. 3, p. 215-227, May 2001.

KNOPP, S.; BYTOF, G.; SELMAR, D. Influence of processing on the content of sugars in green Arabica coffee beans. **European Food Research and Technology**, Berlin, v. 223, n. 2, p. 195-201, Dec. 2005.

MASOUD, W. et al. Yeast involved in fermentation of Coffea arabica in East Africa determined by genotyping and by direct denaturating gradient gel electrophoresis. **Yeast**, Chichester, v. 21, n. 7, p. 549-556, May 2004.

MASOUD, W.; JESPERSEN, L. Pectin degrading enzymes in yeasts involved in fermentation of Coffea arabica in East Africa. **International Journal of Food Microbiology**, Amsterdam, v. 110, n. 3, p. 291-296, Aug. 2006.

MASOUD, W.; KALTOFT, C. H. The effects of yeasts involved in the fermentation of Coffea arabica in East Africa on growth and ochratoxin A (OTA) production by Aspergillus ochraceus. **International Journal of Food Microbiology**, Amsterdam, v. 106, n. 2, p. 229-34, Feb. 2006.

MASSAWE, G.A.; LIFA, S.J. Yeasts and lactic acid bacteria coffee fermentation starter cultures. **International Journal of Postharvest Technology and Innovation**, Genève, v. 2, n. 1, p. 41-82, 2010.

ORGANIZAÇÃO INTERNACIONAL DO CAFÉ. **Do grão à xícara:** aspectos botânicos. Disponível em: <http://www.ico.org/pt/botanical_p.asp>. Acesso em: 24 abr. 2012.

PEE, W. van; CASTELEIN, J. M. The yeasts flora of fermenting robusta coffee. **East African Agricultural and Foresty Journal**, Nairobi, v. 26, n.3, p. 308-310, 1971.

PUERTA-QUINTERO, G. I. Efecto de enzimas pectolíticas en la remoción del mucílago de *Coffea arabica* L., según el desarrollo del fruto. **Cenicafé**, San José, v. 60, n. 4, p. 283-304, 2009.

SAKIYAMA, C. C. et al. Characterization of pectin lyase produced by an endophytic strain isolated from coffee cherries. **Letters in Applied Microbiology**, Oxford, v. 33, n. 2, p. 117-121, Aug. 2001.

SCHWAN, R. F.; SILVA, C. F.; BATISTA, L. R. Coffee fermentation. In: HUI, Y. H. (Ed.).**Handbook of plant-based fermented food and beverage technology**. Boca Raton: CRC, 2012. p. 677-690.

SCHWAN, R. F.; WHEALS, A. E. Mixed microbial fermentations of chocolate and coffee. In: BOEKHOUT, T.; ROBERT,V. (Ed.).**Yeasts in food:** beneficial and detrimental aspect. Hamburg: Behr's Verlag, 2003. p.426-459.

SILVA, C. F. et al. Evaluation of a potential starter culture for enhance quality of coffee fermentation. **World Journal of Microbiology & Biotechnology**, Oxford, v. 29, n.2, p. 235-47, 2013.

SILVA, C. F. et al. Microbial diversity during maturation and natural processing of coffee cherries of *Coffea arabica* in Brazil. **International Journal of Food Microbiology**, Amsterdam, v. 60, p. 251-260, 2000.

SILVA, C. F. et al. Succession of bacterial and fungal communities during natural coffee (*Coffea arabica*) fermentation. **Food Microbiology**, London, v. 25, n. 8, p. 951-957, Dec. 2008.

SOUTHGATE, D.A.T. **Determination of foods carbohydrates**. London: Elsevier Applied Science, 1991. 232 p.

TEIXEIRA, A. A. et al. The science of quality. In: ILLY, A.;VIANI, R. (Ed.).**Espresso coffee**. Rome: Elsevier Academic,1985. p. 197-198.

THOMPSON, S. S.; MILLER, K. B.; LOPES, A. L. Cocoa and coffee. In: DOYLE, M. P.; BEUCHAT, L. R.; MONTVILLE, T. J. (Ed.). **Food microbiology:** fundamentals and frontiers.2nd ed. Washington: ASM, 2001. p.721-733.

UENOJO, M.; PASTORE G. M. Pectinases: aplicações industriais e perspectivas. **Quimica Nova**, São Paulo, v. 30, n. 2, p. 388-394, mar./abr. 2007.

VILELA, D. M. et al. Molecular ecology and polyphasic characterization of the microbiota associated with semi-dry processed coffee (*Coffea arabica L.*). **Food Microbiology**, London, v.27, n. 8, p. 128-135, Dec. 2010.

SEGUNDA PARTE - ARTIGOS

ARTIGO 1 Improvement of coffee beverage quality by using selected yeasts strains during the fermentation in dry process

Artigo Publicado no periódico: *Food Research International*

Improvement of coffee beverage quality by using selected yeasts strains during the fermentation in dry process

Suzana Reis Evangelista[1], Cristina Ferreira Silva [2], Maria Gabriela Pedrozo da Cruz Miguel[2], Cecília de Souza Cordeiro[1], Ana Carla Marques Pinheiro[1], Whasley Ferreira Duarte[2], Rosane Freitas Schwan[1,2]*

[1]Food Sciences Department, Federal University of Lavras, CEP 37200-000, Lavras, MG, Brazil

[2]Biology Department, Federal University of Lavras, CEP 37200-000, Lavras, MG, Brazil

*Corresponding author

[telephone (+55) 35 3829 1614; fax (+55) 35 3829 1100; e-mail: rschwan@dbi.ufla.br].

Running title: Evaluation of the use of starter cultures in coffee fermentation

Abstract

Coffee is an important commercial product to Brazil with its consumption distributed globally. The aim of this work was to evaluate the potencial of yeasts strains as starter cultures for dry fermentation of washed and non - washed coffee beans. Four yeast strains (*Saccharomyces cerevisiae* UFLA YCN727, *Saccharomyces cerevisiae* UFLA YCN724, *Candida parapsilosis* UFLA YCN448 and *Pichia guilliermondii* UFLA YCN731) were inoculated separately in washed and non washed coffee cherries and in the control was not added any of the starter cultures. The fruits inoculated were spread on trays and placed on a terrace until the coffee beans reached 11% of moisture. Samples were collected for evaluation of the persistence of the inoculum by PCR-DGGE, and for chemical composition by HPLC and HS-SPME/GC. Sensory analysis was performed using the Temporal Dominance of Sensations (TDS) methodology. In all tests the yeasts persisted until the end of fermentation. There was no propionic and butyric acids production in concentrations that could compromise the final quality of the beverage. Forty - eight volatile compounds were identified, some were similar for green and roasted coffee. The most abundant class of compounds were

alcohols (11- 27%) followed by furan in roasted grains (~27%), and aldehydes (~13%) in green grains. The coffee inoculated with yeast showed sensations of flavors higher than the control coffee indicating increased sensory quality. The treatment with *C. parapsilosis* UFLA YCN448 showed dominance rate higher (near 1) for the sensation of caramel. In non-washed coffee those sensations were not pleasant in relation to the washed coffee, except when *P. guilliermondii* UFLA YCN731 was inoculated, suggesting that washing the fruit before the fermentation process positively influenced the final product quality. A coffee with special aroma of caramel, herbs and fruits could be produced using the starter cultures *C. parapsilosis* UFLA YCN448 and *S. cerevisiae* UFLA YCN727 in coffee processed by the dry method.

Keywords: Coffee fermentation; starter culture; yeasts; coffee quality

1. Introduction

Coffee is an important commercial product to Brazil with its consumption distributed globally. Nowadays, consumers can choose the most preferred type of beverage from the most full-bodied to the lightest

flavors, organic or conventional, pale to dark, or with fruited flavors or with essences.

After harvesting, coffee fruits are processed to allow a spontaneous or indigenous fermentation to occur. The fermentation can be either a dry or a wet process, and sometimes a combination of both, which is called semi-dry process (Esquivel & Jiménez, 2012; Vilela, Pereira, Silva, Batista, & Schwan, 2010). After harvesting the coffee fruits might be washed or not prior to being spread out in thin layers of 5-8 cm thick on cement patios, where they remain exposed to the sun until they reach 11-12% moisture content. Fermentation of the pulp and mucilage within the fruit occurs during this period which may last up to 20 days which also corresponds to the fruit drying period (Schwan, Silva& Batista, 2012).

Dry processing is characterised by being fully aerobic, which retains a larger concentration of glucose and fructose in the fruits, as they are less consumed in the seeds metabolism (Knopp, Bytof, & Selmar, 2005). These sugars and pectins present in the mucilage will allow microorganisms growth, especially bacteria and yeasts. The access of the epiphytic microorganisms to the pulp can be given by the action of

pectinolytic and cellulolytic enzymes, opening the micropores on the skin-peel-bark due to the loss of water by the opening of the peduncle after harvest.

Coffee quality is a highly complex trait, and depends on physical and sensory qualities as moisture content, defects, bean size, some chemical compounds and preparation of a sample to perform cup tasting (Leroy et al., 2006). The presence of microorganisms may interfere with some of these features. During coffee fermentation some pectinolytic microorganisms are associated with the degradation of the pulp and mucilage (rich in polysaccharides) producing alcohols and acids and other metabolic compounds that interfere in the final beverage quality. Silva et al (2013) studied these characteristics to select microorganisms to be used as culture starters on coffee fermentation.

There are different kinds of coffee beverages characterized by different nuances in terms of body, aroma, acidity and astringency. There are many factors that influence the final beverage, and the action of microorganisms is one of them (Esquivel & Jiménez, 2012). The microbial metabolites produced in this period can diffuse into the grains and influence the beverage final quality. The microbial diversity in this

process is high and several species of bacteria, yeasts, and filamentous fungi have been identified (Silva, Batista, Abreu, Dias, & Schwan, 2008).

The fermentative activity of the microbiota naturally present in coffee fruits has been discussed by some authors (Masoud, Cesar, Jespersen, & Jakobsen, 2004; Masoud, Poll& Kaltoft, 2005; Silva, Schwan, Sousa Dias, & Wheals, 2000; Silva et al., 2013). However, there is still need to understand the influence of specific groups of strains in coffee dry, semi-dry and wet processing and beverage quality. The aim of this work was to use selected yeasts to inoculate natural coffee fermented by dry process in order to improve the final beverage quality.

2. Material and methods

2.1.Reagents

Malic, propionic and citric acid were purchased from Merck (Germany), lactic acid was purchased from Sigma-Chemical (EUA), acetic and succinic acids were purchased from Sigma-Aldrich (Germany), butyric acid was purchased from Riedel-de Haen (Germany).

2.2.Sampling

Coffee cherries of *Coffea arabica* L. var. Acaiá were manually harvested at the mature stage (red cherries) from a farm 750 - 800 m above sea level situated in Lavras in the state of Minas Gerais, Brazil. Half of the harvested coffee was immersed in clean water and immediately transferred to a tray (this fraction was named for washed Coffees). Washed and non-washed fruits were spread (600 g) on a tray in layers of 5 cm and processed using the dry method (coffee cherries were fermented and sun dried until they reached 11-12% of moisture). The ambient temperature ranged from 14.6 to 28.2 °C, and relative humidity from 39 a 72%. Every day, the fruit were scrambled, as usually done, in dry process at farms. Samples were collected aseptically each 4 days, placed in sterile plastic bags, and transferred to the Microbiology Laboratory of the Federal University of Lavras (UFLA) for microbiological and physicochemical analyses.

2.3.Microorganisms used as starters cultured

The yeast isolates used as starters culture were *S. cerevisiae* UFLA YCN727, *S. cerevisiae* UFLA YCN724, *C. parapsilosis* UFLA

YCN448 and *P. guilliermondii* UFLA YCN731 belonging to the Culture Collection of the Microbial Physiology Laboratory at the Biology Department, UFLA, Lavras, MG, Brazil. These yeasts had been previously isolated from coffee fruit (*Coffea arabica* L. var. Acaía) during dry and semi-dry processing and their potential for use as culture starters in coffee fermentation was evaluated in previous work (Silva et al. 2013).

Each inoculums stored in the freezer–80 ºC were reactivated in YEPG medium (ing L^{-1}: glucose20.0, yeast extract 10.0, peptone soy 10.0 and agar 20.0) incubated at 28°C / 150 rpm, in increasing volumes, until they reached approximately 10^8CFU/mL (*C. parapsilopsis* UFLA YCN448; *S. cerevisiae* UFLA YCN724 and UFLA YCN727 and, respectively; *P. guilliermondii* UFLA YCN731). The biomass was harvested after centrifugation and resuspended with 10 mL of distilled water and each treatment was inoculated with one yeast. The control fermentation was carried out without inoculated microorganisms but 10 m L of water was sprayed in order to have the same humidity as the other treatments. All fermentations were carried out in triplicate as a natural process method. Figure 1 shows the methodology flowchart.

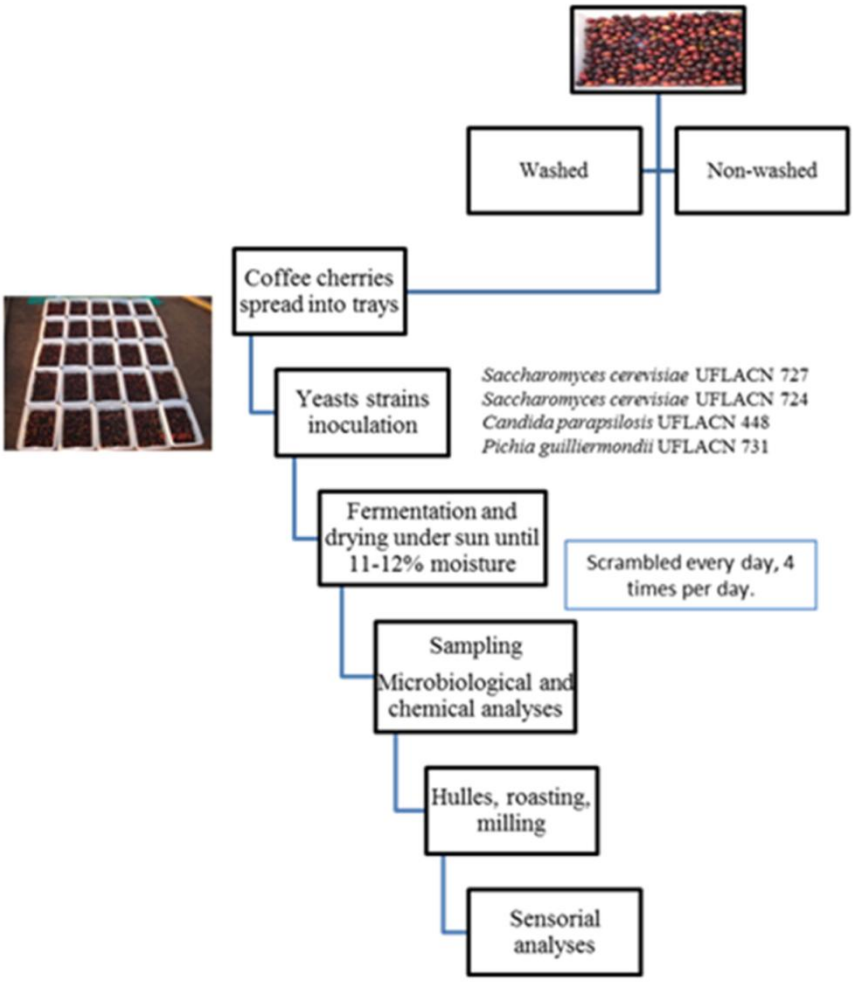

Figure 1. Flow chart of the methodology used.

2.4.Enumeration of microorganisms

Each sample (two grams) was added to a bottle containing 18mL of saline-peptone water (1g L^{-1} bacteriological peptone, Himedia). After mixing for 20 min at 150rpm in an orbital shaker, ten-fold dilutions were

prepared. Microorganisms were counted using two different culture media: PCA (in g L^{-1} tryptone 5; yeast extract 2.5; glucose 1; agar 15) was used as a general medium for the viable bacteria population and YEPG (ing L^{-1}: glucose20.0, yeast extract 10.0, peptone soy 10.0 and agar 20.0) was used for yeasts. Following inoculation, plates were incubated at 28°C for 48 h. After that, the morphological characterisation was done and the population was estimated.

2.5.Microbial community analysis through PCR-DGGE

2.5.1. Total DNA extraction and PCR analysis

Coffee samples from the initial and final fermentation periods were collected to DGGE analysis. Three grams of sample was mixed with 5 ml of Milli-Q water for 10 min, the fluids were centrifuged 100×g for 10 min at 4 °C. The pellet was used for DNA extraction. Total DNA was extracted from samples at two periods of fermentation (initial and end) using the DNA Purification from Tissues protocol [QIAamp DNA Mini Kit (Qiagen, Hilden, Germany)] in accordance with the instructions of the manufacturer. After purification, the samples were PCR-amplified and

analysed in 1% agarose gel and the final samples were stored at -20°C until further use.

The DNA from the bacterial community was amplified with the 338fgc primers (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG-3′) (the GC clamp is underlined) and 518r (5′-ATT ACC GCG GCT GCT GG-3′), which span the V3 region of the 16S rRNA gene (Ovreas, Forney, & Daae, 1997). A fragment of the D1-region of the 26S rRNA gene was amplified with the eukaryotic universal primers NL1GC (5′ CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA TAT CAA TAA GCG GAG GAA AAG-3′) (the GC clamp is underlined) and LS2 (5′-ATT CCC AAA CAA CTC GAC TC-3′), which amplified a fragment of approximately 250 bp (Cocolin, Bisson, & Mills, 2000). PCR was carried out in a final reaction volume of 25 µL containing 0.625 U Taq DNA polymerase (Promega, Milan, Italy), 2.5 µL 10 × buffer, 0.1 mM dNTP, 0.2 mM of each primer, 1.5 mM $MgCl_2$ and 1 µL of extracted DNA. The amplification was performed according to Ramos et al. (2010). Aliquots (3 µL) of the amplification products were analysed by electrophoresis on 1% agarose gels before they were used for DGGE.

2.5.2. PCR-DGGE analysis

The PCR products were separated in polyacrylamide gels [8% (w/v) acrylamide: bisacrylamide (37.5:1)] in 1× TAE buffer with a DCode system apparatus (BioRad Universal Dcode Mutation Detection System, Richmond, CA, USA). Optimal separation was achieved with a 15–55% urea-formamide denaturing gradient for bacteria community and 20–60% for the yeast community (100% correspondent to 7 M urea and 40% (v/v) formamide).

Electrophoresis was conducted at a constant voltage of 130 V for 6 h (bacteria and yeasts and at a constant temperature of 60 °C. Following electrophoresis, the gels were stained with SYBR-Green I (Molecular Probes) (1:10.000 v/v) for 30 min. The images were visualised and photographed using a Transilluminator (LPix®).

2.5.3. DGGE bands sequencing

Selected bands from the PCR–DGGE gels were excised with a sterile blade and placed in 50 μL of sterile Milli-Q water at 4 °C overnight to allow the DNA to diffuse out of the polyacrylamide matrix

and re-amplified with the 338fgc and 518r primers for bacteria and NL1 and LS2 for yeast.

The DNA fragments were purified with a QIAEX II gel extraction kit (Qiagen, Chatsworth, CA, USA) the PCR products were sequenced by UNESP University, (Jaboticabal, São Paulo, Brazil). The sequences were compared with those available in the GenBank database with the BLAST algorithm (National Center for Biotechnology Information, Maryland, USA).

2.6.Analysis of organic acids in coffee

Organic acids (malic, lactic, acetic, butyric, propionic, citric, oxalic, succinic and tartaric acid) were analysed using a high-performance liquid chromatography system Shimadzu, model (Shimadzu Corp., Japan) with a UV detector at 210nm. Three grams of sample was mixed with 5 ml of Milli-Q water for 10 min and the fluids were centrifuged 10000×g for 10 min at 4 °C two times. Samples were microfiltered through a 0.2 µm cellulose acetate filter and directly injected (20 µl) onto the chromatographic column. A Shimpack SCR-101H (7.9 mm×30 cm) column operating at 50 °C was used to achieve chromatographic

separation of water-soluble acids, that were eluted with 100 mM of perchloric acid at a flow rate of 0.6 mL/min. The acids were identified by comparison with retention times of authentic standards. The quantification of compounds was performed using calibration curves constructed with injections (at the same conditions of samples) of different concentrations of standard compounds.

2.7.Analysis of volatile compounds in coffee

2.7.1. Extraction of volatile by headspace-SPME

Coffee sample were macerated with nitrogen for headspace analysis. A Carboxen/poly (dimethylsiloxane) (DVB/CAR/PDMS) type 75 µm SPME fiber (Supelco Co., Bellefonte, PA, USA) was used to extract volatile constituents from the coffee headspace. Two grams of coffee was placed in a 15mL hermetically sealed flask, and heated for 10 min at60°C, to reach sample headspace equilibrium. Then, volatile compounds were extracted by placing the SPME fiber in the headspace for 30 min at 60°C. For compound desorption, the fiber was placed in the GC injection port heated at 230 °C for 5 min.

2.7.2. HS-SPME/GC analysis

The analysis of volatile compounds of green and roasted beans was performed using a gas chromatograph (GC), Shimadzu model 17A equipped with an FID (flame ionisation detector) and a capillary DB wax column (30 m × 0.25 mm i.d. × 0.25 μm) (J&W Scientific, Folsom, Calif., U.S.A.). The oven temperature was maintained at 50 ^{o}C for 5 min, raised to 190 ^{o}C by increments of 3 ^{o}C/min, and then maintained at 190 ^{o}C for 10 min. Injector and detector temperatures were kept at 230 and 240 ^{o}C, respectively. The carrier gas (N_2) was maintained at a flow rate of 1.2 mL/min. Volatile compounds were identified by comparing the retention times of the compounds with those of standard compounds injected under the same conditions (Duarte et al., 2010). The relative percentages of individual compounds were calculated from the total area of volatiles on the chromatograms (Petisca, Pérez-Palacios, Farah, Pinho, & Ferreira, 2013)

2.8.Sensory analysis

The samples were prepared according to the Specialty Coffee Association of America (SCAA, 2012). The coffee grains were roasted in

a Probat roaster, “Probatino”model, having a capacity of 150 g and ground in a Pinhalense ML-1 electric mill. A panel of trained coffee tasters, with Q-Grader Coffee Certificate was employed for this study. Two methodologies were applied to evaluate the sensory characteristics of coffee. The first sensory evaluation was conducted according to SCAA (SCAA, 2012) standards, assessing ten attributes: Fragrance, Flavor, Aftertaste, Acidity, Body, Uniformity, Balance, Sweetness, Cleanliness and Score. After that, the Temporal Dominance of Sensations (TDS) analysis was used (Pineau et al., 2009)**.** The most relevant attributes for describing the temporal evolution of sensations induced by each sample were selected from the main lists by those trained coffee experts. The attributes selected were: chocolate, acidic, bitter, fermented, fruity, herbaceous, caramel, and spicy. The total duration of the proof taste was 20 s. The panelists imbibed the coffee, moved it around in their mouths for 3 s before swallowing it. The evaluation continued until no sensation was perceived at a maximum of 20 s. The act of swallowing the sample facilitated the temporal sensory perception of the product, according to reports of the panelists. Samples coded with three digits were submitted in balanced order (Wakeling & MacFie, 1995). The panelists evaluated

each sample in triplicate. Data were recorded using the SENSOMAKER Software (Nunes & Pinheiro, 2012) and plotted as TDS curve showing, for each sample, the percentage of subjects which selected the attribute as dominant at a specific time, i.e. the dominance rate (Pineau et al., 2009).

2.9.Statistical analysis

Data of volatiles compounds and sensory analysis were statistically analysed by principal component analysis (PCA) using SENSOMAKER Software (Nunes&Pinheiro, 2012). An *m* x *n* matrix was built with the relative areas of the *n* identified chromatographic peaks for the *m* samples, for volatiles compounds. An *m* x *n* matrix was built with the values of the *n* attributes for the *m* samples, for sensory analysis. The data were auto scaled.

3. Results and discussion

3.1.Yeast population during the coffee fermentation

Total microbial and yeast counts showed no significant differences during the fermentation processes with different strains inoculated in washed and non-washed coffee cherries (Figure 2). However, it was

possible to observe that the microbial population was higher in non-washed coffees, which was already expected due to the physical removal of microorganisms during washing.

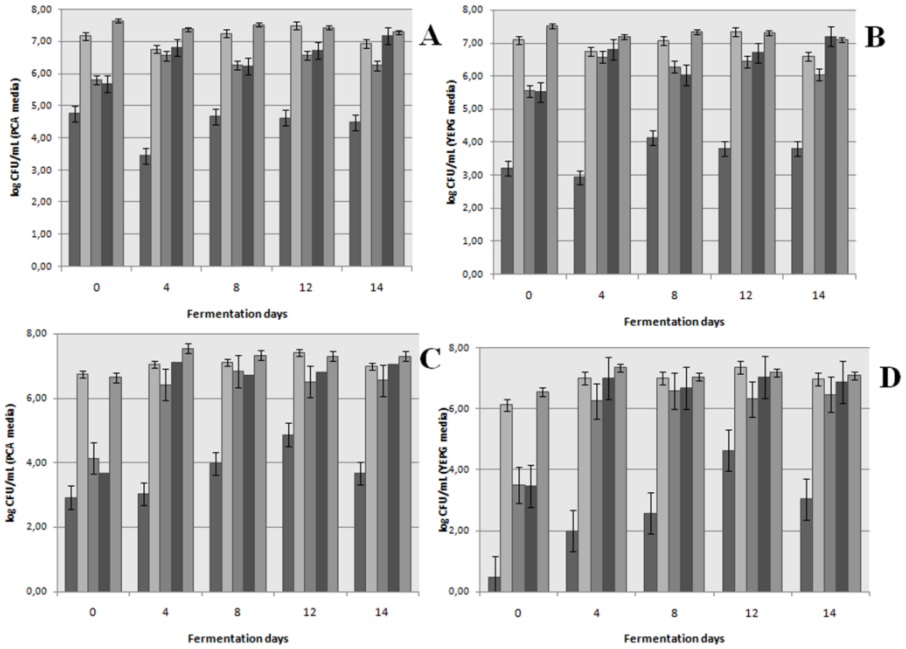

Figure 2. Total bacteria population (PCA media-A and C) and yeast population (YEPG media-B and D) from the inoculation of 4 yeast strains selected during the fermentation of dry-processed, non-washed coffee (A and B) and washed coffee (C and D). Control ( ), *C. parapsilosis* UFLA YCN448 ( ), *S. cerevisiae* UFLA YCN724 ( ), *S. cerevisiae* UFLA YCN727 ( ), *P. guilliermondii* UFLA YCN731 ( ).

The bacteria population was 1.57 times higher than yeasts in the treatments where coffee cherries were washed before fermentation (Figure 2). The identification of bacteria and yeasts population in dry processed coffee has been reported by Silva et al. (2000; 2008). Usually, bacteria and yeasts predominate in the early stages of fermentation due to the high content of water present in the fruits (Silva, Batista, Abreu, Dias, & Schwan, 2008). The yeast population was higher in the treatments where selected strains were inoculated than in the control. These results showed that the inoculated strains were able to compete with the epiphytic microorganisms already present in coffee fruits, and to use the coffee fruit pulp as substrate. Another result was that they could persist throughout the fermentation process.

Analysing each treatment, it was observed that the population of treatment inoculated with *C. parapsilosis* UFLA YCN448 in non-washed coffee was constant during the fermentation, howevershowed growth of 1 log cycle in washed coffee. *S. cerevisiae* UFLA YCN724 and UFLA YCN727 treatment showed no difference in population between them, although it was below that of *C. parapsilosis* UFLA YCN448 treatment. The *P. guilliermondii* UFLA YCN731 treatment showed better growth in

the first 4 days of fermentation in washed coffee. The four yeast strains could be indicated as culture starters for coffee dry processing considering aspects of persistence and dominance (Figure 2).

3.2.DGGE analysis

The starter cultures were detected throughout the fermentation process (Figure 3) in DGGE gels. *Candida parapsilosisis* (1 isolate) and *P. guilliermondii* (1 isolate) and *S. cerevisiae* (two isolates) showed a different band pattern identified as bands 1 and 2 (*C. parapsilosis*), 3 and 4 (*P. guillliermondii*), and 5 and 6 (*S. cerevisiae*). The multiple banding patterns of the strains may be due to sequence heterogeneity generating multiple copies of the 18S rRNA (Nübel, Garcia-Pichel, Kuhl & Muyzer, 1999).

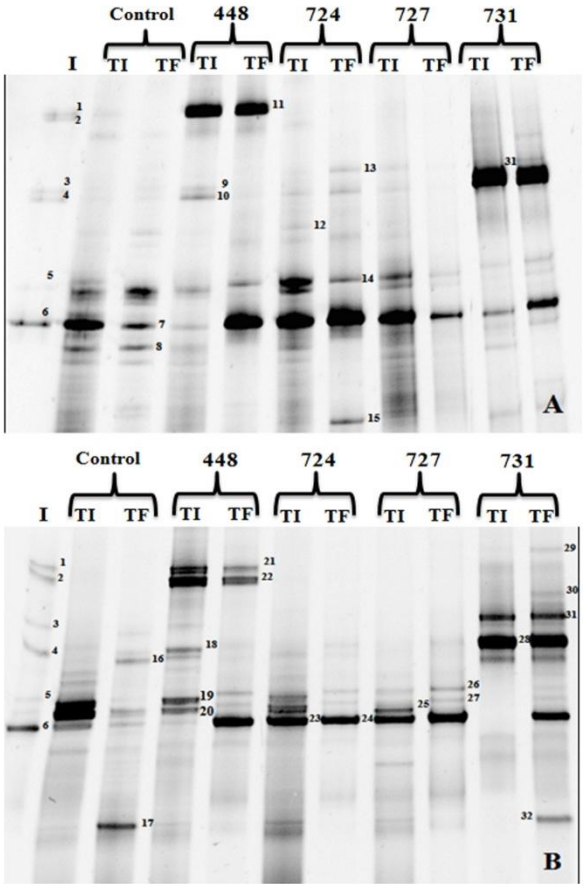

Figure 3. DGGE analyses of yeasts communities found in the beginning (TI) and at the end (TF) of the fermentation process of washed (A) and non-washed coffee (B). I= mix of the strains inoculates in fruit. The numbers over the spots represent the yeast strains: *C. parapsilosis* UFLA YCN448, *S. cerevisiae* UFLA YCN724, *S. cerevisiae* UFLA YCN727, *P. guilliermondii* UFLA YCN731. The identities of the bands and acess number are: 1, 2, 11, 21,22= *Candida parapsilosis* (FJ 009291); 3, 4, 9, 10, 18, 28, 31= *Pichia guilliermondii* (EF 197951)*;* 5, 6, 7, 14, 23, 24= *Saccharomyces cerevisiae* (EU 649672); 8,12, 26, 27= *Mitchella repens* (JQ 417238); 13, 16= *Pichia kluyveri* (JQ 219339); 15, 17, 29, 32= *Torulaspora delbrueckii* (EU 879961); 19= *Hanseniaspora opuntiae* (DQ 872866); 20, 25= *Debaryomyces hansenii* (FJ 475230); 30= unidentified. Similarity above 99%.

S. cerevisiae was detected from samples where they were inoculated and also in the control samples, suggesting that this strain is epiphytic on coffee fruits and present in the environmental conditions during the fermentation process (Figure 3A). All four strains tested here were able to persist until the end of the fermentation period. *S. cerevisiae* UFLA YCN724 strain did not show a reduction of its population observing the intensity of the band in PCR-DGGE (Figure 3A). This observation was because DGGE is a semi-quantitative method and the initial concentration of the population of each inoculum was known. *Toruslapora delbrueckii* (band 15) and *P. kluyveri* (band 13) were found at the beginning of fermentation (lower population compared to yeasts used as inoculum) but had their populations reduced at the final period of fermentation.

Yeasts were also found in samples from the control of non-washed coffee. The presence of *Hanseniaspora opuntiae* (band 19*)*, *Debaryomyces hansenii* (bands 20 and 25), *Torulaspora delbrueckii* (bands 17 and 32) and *P. kluyveri* (band 16) (Figure 3B) was detected. Bands 5 and 6, identified as *S. cerevisiae,* were detected in all fermentations. *S. cerevisiae* has been reported as one of the naturally

found yeasts in coffee fermentation (Silva, Batista, Abreu, Dias, & Schwan, 2008).

All inoculated strains were detected in all fermentation periods, which indicated that they were able to compete with the great diversity of naturally present microbe species in coffee fruits. The coffee fruits presented drastic changes in the physicochemical composition of the fruit such as the decrease of nutrients and the reduction of water activity (0.6) at the end of the fermentation period.

During fermentation with UFLA YCN724 and UFLA YCN727 strains (Figure 3 B) it was possible to observe the presence of *T. delbrueckii* in some samples, but it did not persist until the end of the fermentation, probably due to competition for nutrients with the inoculated strains. At the end of the fermentation inoculated with UFLA YCN731 strain it was also possible to detect *T. delbrueckii* (band 32) and *S. cerevisiae* (band 24).

The bacteria diversity present was generally greater than the yeast diversity (Figure 4). This finding has also been reported by Vilela et al. (2010). *Erwinia billingiae* (bands 2 and 10), *Leuconostoc mesenteroides* (bands 3 and 9) *Halospirulina* sp (bands 4 and 7), *Pantoea agglomerans*

(bands 11), *Pantoea dispersa* (band 12), uncultured *Pantoea* (band 13) were the bacteria found during coffee fermentation inoculated with yeasts. The presence of several species of bacteria was easily noted in the DGGE gel (Figure 4B) of samples from coffee with or without inoculum. The profile found in microbial fermentations with UFLA YCN448 and UFLA YCN724 strains were very similar to the profile of the control sample (Figure 4B), suggesting that these inoculum were not able to inhibit bacteria. For all samples, regardless of the inoculated strain, it was possible to observe the presence of the bacteria *Pantoea* sp (band 17) and *Pantoea brenneri* (band 18) at the end of the fermentation process.

One of the DGGE bands was identified as *Mitchella repens* (bands 8, 12, 26, and 27), which is a plant belonging to the *Rubiaceae family*. The identification of a plant was possible due to the use of universal primer for the eukaryota; consequently the DNA of the plant was also amplified. This specie was also identified by Masoud et al. (2004) during coffee fermentation in Tanzania. These authors reported that *M. repenses* is a plant that belongs to the botanical genus *Mitchella* of the family Rubiaceae, to which the genus *Coffea* belongs.

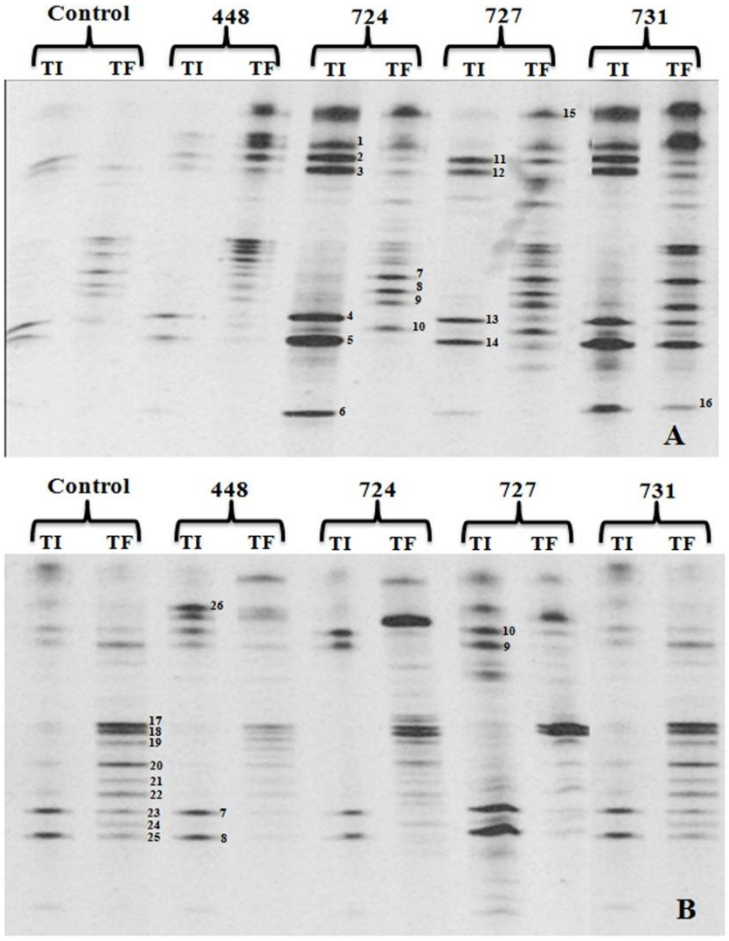

Figure 4. DGGE analyses of bacteria communities found in the beginning (TI) and at the end (TF) of the fermentation process of washed (A) and non-washed coffee (B). The identities of the bands and acess number are: 1= Uncultured bacterium(GU 301232), 6, 16= Uncultured bacterium (GU 301189); 2, 10= *Erwinia billingiae* (JQ 353778); 3, 9= *Leuconostoc mesenteroides* (GU 458344); 4,7,23= *Halospirulina* sp (JX 912466); 5,8,25= unidentified; 11, 20= *Pantoea agglomerans* (JN 645943); 12, 21= *Pantoea dispersa* (JQ 659939); 13, 22= *Pantoea* sp (FR 692005); 14, 24= *Pectobacterium* sp (FJ 784694); 15= *Cryptosporidium environmental* (JQ 178286); 17= *Pantoea* sp (EU 741017); 18= *Pantoea brenneri* (HF 585161); 19= *Pantoea eucrina* (JX 865456). Similarity above 98%.

3.3.HPLC analysis

Coffee seeds have all the precursors needed to generate typical flavor and aroma during the roasting operation (Joët et al., 2010).However, the growth of microorganisms during the processing stages may confer additional flavor notes due to metabolites produced by fermentation, and their subsequent potential to migrate into the seed.

Tartaric and butyric acids were not detected in none of the analysed samples. The absence of butyric acid indicates that the coffee fruits did not undergo fermentation.The acetic (maximum concentration 10.52 g/Kg) and propionic (2.09 g/Kg) acids (Table 1) are important microbial metabolites in the final quality of the coffee beverage (Bertrand et al., 2012; Lopez, Bautista, Moreno & Dentan, 1989). These acids were found when *S. cerevisiae* and *P. Guilliermondii* strains were inoculated, or when *S. cerevisiae* was naturally present (Figure 3). Acetic, malic and citric acids contribute to the acidity of the final beverage (Buffo & Cardelli-freire, 2004). Usually, the acetic acid is easily accumulated in warm environmental temperatures (T > 22 ºC) (Bertrand et al., 2012). The temperature during the days of fermentation was highly variable, with values between 14.6 and 28.2 °C (data not shown), which probably did not show a positive correlation with the accumulation of acetic acid.

It is important to observe that the simple presence of the specific organic acids is not responsible for the interference in the final quality but their concentrations might affect the coffee quality. In this case, the higher concentration of acetic acid observed was 10.52 g/Kg in the pulp+seed sample in non-washed coffee inoculated with *P. guilliermondii* UFLA YCN731 but the concentration was lower inside the seed (2.07 g/Kg). All tested samples showed low concentrations of propionic acid (0.07-2.09 g/Kg). These acids should not be present in concentrations higher than 1 mg/mL for a good coffee beverage (Lopez, Bautista, Moreno & Dentan, 1989).

Table 1
Organic acids present in coffee inoculated with yeast and the control, in the natural washed and natural non-washed coffee in the begining and in the end of fermentation process. The organic acids were evaluated in pulp + seed and seed.

		Organic acids (g/Kg)											
Fermentation samples	Fermentation Time (days)	**PULP + SEED**						**SEED**					
		Citric	Malic	Succinic	Lactic	Acetic	Propionic	Citric	Malic	Succinic	Lactic	Acetic	Propionic
No-washed coffee													
Control[a]	0	0.41	2.78	6.46	0.04	nd	nd	0.02	1.04	1.59	0.02	nd	nd
Control	14	1.87	3.14	17.70	nd	0.08	nd	0.36	0.70	3.06	nd	nd	nd
UFLA YCN448[b]	0	0.06	4.48	5.35	nd	nd	nd	0.07	2.03	1.82	nd	nd	nd
UFLA YCN448	14	0.77	2.00	4.87	1.37	nd	nd	0.07	0.55	1.24	0.20	nd	nd
UFLA YCN724[c]	0	0.67	4.39	7.82	0.01	nd	nd	0.06	1.12	2.00	nd	nd	nd
UFLA YCN724	14	1.34	1.81	4.61	nd	nd	nd	0.59	0.90	2.21	nd	nd	nd
UFLA YCN727[d]	0	0.11	3.83	3.41	nd	7.34	0.85	0.05	1.48	1.73	nd	nd	nd

Table 1 (continued)

UFLA YCN727	14	2.07	2.43	6.54	nd	4.86	2.09	0.33	0.49	1.20	0.02	nd	nd
UFLA YCN731[e]	0	0.32	3.92	6.11	0.05	10.52	0.40	0.09	0.41	0.49	0.01	2.07	0.11
UFLA YCN731	14	2.03	1.41	5.36	nd	2.99	1.55	0.39	0.49	1.48	nd	1.46	0.43
Washed coffee													
Control	0	0.25	4.76	5.52	nd	nd	nd	0.02	0.97	0.89	0.02	nd	nd
Control	14	0.82	2.80	4.76	nd	nd	nd	0.22	1.01	2.05	nd	nd	nd
UFLA YCN448	0	0.43	4.22	3.20	0.01	nd	nd	0.15	0.34	0.11	nd	nd	nd
UFLA YCN448	14	1.43	3.40	4.42	0.06	nd	nd	0.29	1.34	1.93	0.11	nd	nd
UFLA YCN724	0	nd	2.63	1.55	nd	nd	nd	0.05	1.34	0.75	nd	nd	nd
UFLA YCN724	14	1.07	4.03	9.32	0.14	nd	nd	0.25	0.57	0.90	0.03	nd	nd
UFLA YCN727	0	0.14	1.31	1.07	nd	2.10	0.14	0.10	0.79	0.91	nd	nd	nd
UFLA YCN727	14	1.38	1.87	4.81	0.04	4.95	1.77	0.26	0.72	2.52	0.01	nd	nd
UFLA YCN731	0	0.55	2.60	2.47	nd	7.68	0.28	0.16	0.43	0.55	nd	1.63	0.07
UFLA YCN731	14	2.08	2.11	7.75	nd	7.70	1.46	0.41	0.51	1.44	nd	2.27	0.36

The samples show a standart deviation around 0.02.

[a]Control= without inoculums.

[b]UFLA YCN448 = *Candida parapsilosis* UFLA YCN448.

[c]UFLA YCN724 = *Saccharomyces cerevisiae* UFLA YCN724.

[d]UFLA YCN727 = *Saccharomyces cerevisiae* UFLA YCN727.

[e]UFLAYCN731= *Pichia guilliermondii* UFLA YCN731.

[f]nd = not detectable.

3.4.GC analysis

GC was done to evaluate the chemical composition of the volatiles from the fermentation and roasting. Forty - eight compounds (Tables 2 and 3) including hydrocarbons (1 compost), ketones (3), alcohols (17), aldehydes (5) and acids (5) linked from post-harvest processing, esters (11), phenols (1) and furans (5) from thermal origin were detected. Alcohols were the most abundant compounds (11- 27%) followed by furan in roasted grains (~27%) and aldehydes (~13%) in green grains (Tables 2 and 3). Esters are important compounds from a sensory point of view present in green coffee or which are produced during post-harvest processing (Cantergianai et al., 2001). However, higher concentrations of esters might confer over-fermented flavor. In our work, only 3.89% of the total compounds were esters in green coffee.

Table 2
Relative percentage of volatile compounds extracted from green and roasted non-washed coffee.

	%									
	Green bean	**Roasted bean**	**Green bean**	**Roasted bean**	**Green bean**	**Roasted bean**	**Green bean**	**Roasted bean**	**Green bean**	**Roasted bean**
Compounds/ Yeast	**Control[a]**	**Control[a]**	**448[b]**	**448[b]**	**724[c]**	**724[c]**	**727[d]**	**727[d]**	**731[e]**	**731[e]**
Acetal										
1,1-dietoxyethane	0.14	0.47	0.06	0.75	0.03	0.72	0.03	0.63	0.05	0.73
Ketones										
2,3-butanedione	0.58	0.09	0.29	0.08	0.25	0.11	0.35	nd	0.33	nd
2-nonanone	nd[f]	1.56	nd	2.20	nd	1.93	nd	1.59	nd	2.14
Verbenone	nd	1.18	nd	1.52	nd	1.35	nd	1.01	nd	1.42
Total Ketones	0.58	2.83	0.29	3.80	0.25	3.39	0.35	2.60	0.33	3.56
Alcohols										
Methanol	0.46	1.90	0.33	2.28	0.22	2.38	0.23	1.78	0.23	2.07
1-propanol	0.51	0.07	0.37	0.08	0.63	0.07	0.38	0.07	0.46	0.06
2-methyl-1-propanol	nd	nd	nd	nd	nd	nd	nd	nd	0.08	nd
1-butanol	1.05	0.03	1.73	0.04	0.40	0.02	0.65	0.05	0.27	nd
3-methyl-1-butanol	nd	nd	0.37	nd	nd	nd	0.23	nd	0.19	nd
1-pentanol	nd	5.01	nd	6.22	nd	5.77	nd	5.66	nd	6.17
2-heptanol	1.12	2.52	0.38	3.25	0.70	2.81	0.30	2.55	0.33	2.90
3-mehtyl-1-pentanol	0.23	nd	0.73	nd	0.68	nd	0.46	nd	0.63	nd
1-hexanol	nd	nd	nd	nd	nd	nd	nd	0.02	nd	nd

Table 2 (continued)

Linalool	4.98	0.29	5.35	0.54	4.60	0.72	6.97	0.90	8.12	0.67
1,2-propanediol	nd	0.27	nd	0.26	nd	0.34	nd	0.28	nd	0.32
a-Terpeniol	nd	0.05	nd	nd	nd	0.04	nd	nd	nd	nd
b-Citronellol	nd	0.23	nd	0.20	nd	0.17	nd	0.17	nd	0.20
Geraniol	nd	0.05	nd	0.03	nd	0.04	nd	nd	nd	nd
2-phenylethanol	0.45	0.11	1.67	0.17	1.02	0.29	1.85	0.25	1.84	0.24
Menthol	nd	0.11	0.21	nd	nd	nd	nd	0.10	nd	nd
Total Alcohols	8.81	10.64	11.14	13.07	8.25	12.65	11.07	11.83	12.15	12.63
Aldehydes										
Acetaldehyde	1.76	0.59	1.61	0.52	1.12	0.47	1.09	0.42	1.28	0.54
Butyraldehyde	4.73	0.05	9.98	0.27	18.70	0.44	14.82	0.80	10.75	0.34
Hexanal	0.28	nd	0.15	nd	0.16	nd	0.09	nd	0.23	nd
Octanal	nd	0.40	nd	0.26	nd	0.39	nd	0.51	nd	0.42
Decyl aldehyde	nd	nd	nd	0.38	nd	0.35	nd	0.26	nd	0.35
Total Aldehydes	6.77	1.04	11.74	1.43	19.98	1.65	16	1.99	12.26	1.65
Acids										
Propionic acid	nd	nd	nd	nd	nd	0.33	nd	nd	nd	nd
Isobutyric acid	nd	10.32	nd	9.25	nd	8.72	nd	8.97	nd	9.33
Butyric acid	nd	0.10	nd	0.11	nd	0.11	nd	0.10	nd	0.10
Hexanoic acid	nd	0.06	nd	0.09	0.44	0.09	0.50	0.09	0.18	0.08
Decanoic acid	nd	0.14	nd	0.19	nd	0.16	nd	nd	nd	0.08
Total Acids	nd	10.62	nd	9.64	0.44	9.41	0.5	9.16	0.18	9.59
Esters										
Ethyl acetate	0.06	0.02	nd	0.01	nd	0.01	nd	0.02	nd	0.01
Propyl acetate	2.49	0.83	1.09	0.66	0.90	0.71	0.54	0.59	0.92	0.67
Isobutyl acetate	0.44	nd	0.33	nd	0.28	nd	0.22	nd	0.20	nd
Ethyl butyrate	1.03	0.07	1.73	0.08	1.89	0.08	1.30	0.07	1.51	0.06
Isoamyl acetate	nd	nd	nd	nd	1.03	nd	1.43	nd	0.80	nd

Table 2 (continued)

Ethyl lactate	nd	nd	nd	nd	nd	nd	nd	nd	0.18	nd
Ethyl octanoate	nd	0.01	nd	0.02	nd	0.02	nd	0.02	nd	nd
Diethyl malonate	nd	0.36	nd	0.37	nd	0.39	nd	0.33	nd	nd
Phenyl acetate	nd	0.32	nd	0.34	nd	0.31	nd	0.34	nd	0.35
Propyl butyrate	nd	0.07	nd	nd	nd	0.02	nd	nd	nd	0.02
Diethylsuccinate	nd	0.02	nd	nd	nd	nd	nd	nd	nd	nd
Total Esters	4.02	1.70	3.15	1.48	4.1	1.54	3.49	1.37	3.61	1.11
Phenols										
Guaiacol	nd	0.39	nd	0.23	nd	0.39	nd	0.20	nd	0.20
Furans										
Furfuryl alcohol	nd	12.40	nd	10.76	nd	9.81	nd	12.27	nd	11.22
Furfuryl acetate	nd	3.67	nd	3.08	nd	3.72	nd	4.33	nd	3.58
Furfuryl propionate	nd	0.31	nd	0.21	nd	0.26	nd	0.29	nd	0.33
Furfural	nd	14.39	nd	13.80	nd	13.01	nd	10.81	nd	11.93
5-methylfurfural	1.19	0.01	0.76	nd	0.74	0.01	0.52	nd	0.51	nd
Total Furans	1.19	30.77	0.76	27.85	0.74	26.81	0.52	27.7	0.51	27.06
Total GC area	59223	521920	61355	431442	108068	487511	76741	537204	103307	482993

[a]Control= without inoculums.
[b]UFLA YCN448 = *Candida parapsilosis* UFLA YCN448.
[c]UFLA YCN724 = *Saccharomyces cerevisiae* UFLA YCN724.
[d]UFLA YCN727 = *Saccharomyces cerevisiae* UFLA YCN727.
[e]UFLAYCN731= *Pichia guilliermondii* UFLA YCN731.
[f]nd = not detectable.

Table 3
Relative percentage of volatile compounds extracted from green and roast whased coffee

	%									
	Green bean	**Roasted bean**	**Green bean**	**Roasted bean**	**Green bean**	**Roasted bean**	**Green bean**	**Roasted bean**	**Green bean**	**Roasted bean**
Compounds/ Yeast	**Control[a]**	**Control[a]**	**448[b]**	**448[b]**	**724[c]**	**724[c]**	**727[d]**	**727[d]**	**731[e]**	**731[e]**
Acetal										
1,1-dietoxyethane	0.17	0.50	0.02	0.39	0.02	0.54	0.01	0.43	0.02	0.77
Ketones										
2,3-butanedione	0.29	0.09	0.12	0.06	0.14	0.07	0.08	0.09	0.17	nd
2-nonanone	nd[f]	1.48	nd	1.46	nd	1.58	nd	1.35	nd	1.41
Verbenone	nd	0.98	nd	0.90	nd	0.98	nd	0.85	nd	0.86
Total Ketones	0.29	2.55	0.12	2.42	0.14	2.63	0.08	2.29	0.17	2.27
Alcohols										
Methanol	0.45	1.72	0.12	1.60	0.13	1.68	0.18	1.60	0.07	1.88
1-propanol	1.35	0.05	0.80	0.05	0.83	0.06	0.83	0.07	0.64	0.06
1-butanol	0.57	0.03	nd	nd	nd	0.02	nd	nd	0.12	nd
1-pentanol	nd	5.94	nd	5.22	nd	6.69	nd	5.27	nd	5.85
2-heptanol	0.79	2.77	0.39	2.54	nd	2.94	0.28	2.71	nd	2.61
3-mehtyl-1-pentanol	0.43	nd	0.30	nd	0.53	nd	0.46	nd	0.42	nd
1-hexanol	nd	nd	nd	0.03	nd	nd	nd	0.01	nd	nd
Trans-3-hexen-1-ol	nd	0.27	nd	0.23	nd	0.22	nd	0.24	nd	0.21
Linalool	6.30	0.37	3.92	0.91	3.87	0.76	4.96	1.18	4.60	0.89
1,2-propanediol	nd	0.24	nd	0.30	nd	0.34	nd	0.28	nd	0.36
b-Citronellol	nd	0.24	nd	0.19	nd	0.19	nd	0.18	nd	0.19
2-phenylethanol	0.77	0.12	1.05	0.21	1.05	0.18	1.38	0.23	1.20	0.17
Menthol	nd	0.10	nd	0.07	nd	0.07	nd	nd	0.14	0.08

Table 3 (continued)

Total Alcohols	10.66	11.85	6.58	11.35	6.41	13.15	8.09	11.77	7.19	12.3
Aldehydes										
Acetaldehyde	2.17	0.51	1.33	0.38	0.85	0.33	0.76	0.30	1.12	0.47
Butyraldehyde	3.23	0.10	12.31	0.75	10.60	0.56	17.05	0.89	14.28	0.36
Hexanal	nd	nd	nd	nd	0.06	nd	nd	nd	nd	nd
Octanal	nd	0.49	nd	0.58	nd	0.33	nd	0.34	nd	0.58
Decyl aldehyde	nd	0.25	nd	0.24	nd	0.25	nd	0.20	nd	0.24
Total Aldehydes	5.4	1.35	13.64	1.95	11.51	1.47	17.81	1.73	15.4	1.65
Acids										
Isobutyric acid	nd	9.55	nd	8.22	nd	8.12	nd	8.03	nd	9.48
Butyric acid	nd	0.10	nd	0.09	nd	0.10	nd	0.08	nd	0.10
Hexanoic acid	nd	nd	nd	0.09	nd	0.08	nd	0.08	nd	0.08
Decanoic acid	nd	0.05	nd	nd	nd	nd	nd	nd	nd	nd
Total Acids	nd	9.7	nd	8.4	nd	8.3	nd	8.19	nd	9.66
Esters										
Ethyl acetate	0.07	0.02	nd	0.00	nd	0.01	nd	0.01	nd	0.01
Propyl acetate	2.44	0.79	0.57	0.67	0.56	0.65	0.26	0.59	0.51	0.71
Isobutyl acetate	0.34	nd	0.17	nd	0.16	nd	0.12	nd	0.18	nd
Ethyl butyrate	1.09	0.05	3.11	0.04	3.77	0.08	2.63	0.05	3.17	0.07
Isoamyl acetate	nd	nd	nd	nd	nd	nd	1.47	nd	nd	nd
Ethyl octanoate	nd	0.02	nd	0.02	nd	0.02	nd	0.02	nd	0.04
Diethyl malonate	nd	0.21	nd	0.32	nd	0.18	nd	0.26	nd	0.25
Phenyl acetate	nd	0.38	nd	0.36	nd	0.40	nd	0.41	nd	0.42
Propyl butyrate	nd	0.03	nd	0.03	nd	0.04	nd	0.04	nd	0.04
Diethylsuccinate	nd	0.03	nd	nd	nd	0.04	nd	nd	nd	0.04
Total Esters	3.94	1.53	3.85	1.44	4.49	1.42	4.48	1.38	3.86	1.58
Phenols										
Guaiacol	nd	0.21	nd	0.21	nd	0.19	nd	0.19	nd	0.19

Table 3 (continued)

Furans										
Furfuryl alcohol	nd	15.49	nd	13.56	nd	14.02	nd	14.14	nd	13.89
Furfuryl acetate	nd	3.97	nd	3.94	nd	5.02	nd	4.56	nd	4.96
Furfuryl propionate	nd	0.24	nd	0.18	nd	0.30	nd	0.25	nd	0.27
Furfural	nd	9.99	nd	9.96	nd	8.02	nd	7.61	nd	9.15
5-methylfurfural	0.89	0.01	0.42	nd	0.39	nd	0.31	nd	0.48	nd
Total Furans	0.89	29.7	0.42	27.64	0.39	27.36	0.31	26.56	0.48	28.27
Total GC area	48789	642420	85915	550900	114407	623276	118141	620499	107172	597224

[a]Control= without inoculums.
[b]UFLA YCN448 = *Candida parapsilosis* UFLA YCN448.
[c]UFLA YCN724 = *Saccharomyces cerevisiae* UFLA YCN724.
[d]UFLA YCN727 = *Saccharomyces cerevisiae* UFLA YCN727.
[e]UFLAYCN731= *Pichia guilliermondii* UFLA YCN731.
[f]nd = not detectable.

More than 800 volatile compounds were detected in roasted and green coffees (Farah, Monteiro, Calado, Franca & Trugo, 2006), but not all of them are responsible for coffee flavor (Bertrand et al., 2012). The majority of the compounds detected in green coffee persist during roasting, and some compounds are precursors of flavor compounds during roasting as sucrose and amino acids (Mailard reaction), carbohydrates, chlorogenic acids, nitrogen-containing substances, and lipids (Yeretzian, Jordan, Badoud&Lindinger, 2002).Two factors contribute to the formation of volatile compounds in coffee: microbial metabolites formed during fermentation, and the compounds of the inherent grains (Yeretzian, Jordan, Badoud&Lindinger, 2002). These factors can be very variable in different regions of coffee production. The composition and concentration of volatiles may be influenced by the environment, the variety of the plant, the chemistry of soil, altitude and even storage conditions (Yeretzian, Jordan, Badoud&Lindinger, 2002). Bertrand et al. (2012) observed that climatic conditions, such as temperature, interfere with the formation of volatile compounds in coffee. In places with high environmental temperatures (20-25 °C), there is predominance of earthy and green aromas, and in regions showing mild temperatures (14-16 ºC),

the dominance of fruity aroma and overall quality is generally observed (Bertrand et al., 2012).

Some compounds were detected only in green beans and in grains after roasting, while others were detected in both (Tables 2 and 3). Of the compounds tested, 2,3-butanedione, 3-methyl-1 - butanol, linalool, 1,3-butanediol, 2-phenylethanol, hexanal, nonanal, hexanoic acid and guaiacol were correlated with lower quality of the beverage (Bertrand et al., 2012; Toci & Farah, 2008; Guyot, Cros & Vincent, 1982).

Acetoin, acetaldehyde, isoamyl acetate and furans are compounds that confer a caramel, floral and fruity flavor to the final beverage (Sanz, Maeztu, Zapelena, Bello & Cid, 2002; Czerny & Grosch, 2000). Other compounds had antioxidant action such as furans and acetic acid (Yanagimoto, Lee, Ochi & Shibamoto, 2002; 2004; Fuster, Mitchell, Ochi & Shibamoto, 2000) and antifungal activity as isobutyl acetate (Masoud, Poll& Kaltoft, 2005).

Comparing the results of washed and non-washed coffee, a difference in the concentration of 18 compounds (Table 2 and 3) was observed, and that in general, the-non washed coffee presented these compounds at higher concentrations. Hexanal is a compound that

characterise's the typical odor of green beans before the roasting process (Czerny & Grosch, 2000) and it was detected only in non-washed coffee. Some of these compounds characterise the loss of sensory quality and might identify which defect is present, for example, 2,3-butanedione, 3-methyl-butanol, linalool and hexanal characterise the insect damage; high concentration of propionic acid is related to over fermentation; hexanoic acid is found only in roasted coffee (Toci & Farah, 2008). The two compounds that impart pleasant fruit flavor to the final beverage were decanoic acid and acetaldehyde, which were found in higher concentrations in non-washed coffee.

The PCA analysis allowed the correlation between some volatile compounds and the four treatment inoculated with yeasts in green beans and after roasting. Each axis of the graph shows the percentage of total explained variation (Figures 5A and B). The Figure 5A shows that two principal components explained 54.77 and 20.06% of total variance. The Control was characterized by the presence of 2-heptanol, 2,3-butanedione, hexanal, propyl acetate, 5-methylfurfural, 1,1-dietoxyethane, isobutyl acetate, methanol. Among the others treatments, UFLA YCN727 and UFLA YCN731 were grouped in the positive part of the PC1 and

characterized by linalool, ethyl butyrate. Compared to the roasted coffee (Figure 5C), was observed that Control treatment kept the profile of volatile compounds, while the others treatments presented a different behavior concerning to the volatile profile. A similar behavior (as described above) was observed for Control treatment in Figure 5B (PC1 36.61% of total variance; PC2 31.53% of total variance) and 5D (PC1 42.36% of total variance; PC2 29.08% of total variance).

Depending on the processing (washed and non-washed), the inoculated yeasts behaved differently in both green and roasted coffee; e.g. the UFLA YCN724 and UFLA YCN448 treatment for non-washed roasted beans (Figure 5B) were grouped together and characterized by ethyl butyrate, 1-propanol, guaiacol, propionic acid. The same treatment were plotted separately (Figure 5D) for washed roasted beans and correlated with different volatile compounds (except for the UFLA YCN724 correlated by ethyl butyrate). Comparing washed and non-washed coffee, it was possible to observe that the volatile profile was quite different; showing that washing the coffee changed the final characteristics of the product.

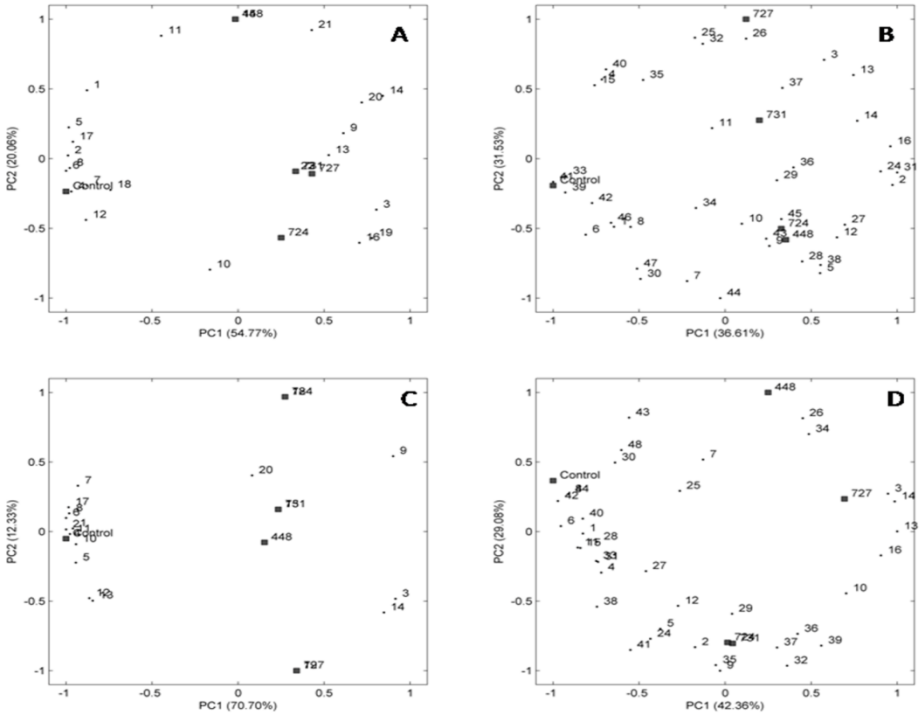

Figure 5. Principal component analysis loadings plot of the following volatile compounds during the fermentation coffee by dry process in non-washed (A- green bean and B- roasted bean) and washed (C- green bean and D- roasted bean) coffee fruits inoculated with the UFLA YCN448, UFLA YCN724, and UFLA YCN727 and UFLA YCN731 yeast strains and Control (no yeast inoculated): 1- Acetaldehyde, 2- 1,1-dietoxyethane, 3- Butyraldehyde, 4- Ethyl acetate, 5- Methanol, 6- Propyl acetate, 7- 2,3-butanedione, 8- 5-methylfurfural, 9- Ethyl butyrate, 10- 1-propanol, 11- 1-butanol, 12- 2-heptanol, 13- Linalool, 14- 2-phenylethanol, 15- Menthol, 16- Hexanoic acid 17- Isobutyl acetate, 18- Hexanal, 19- Isoamyl acetate, 20- 3-mehtyl-1-pentanol, 21- 3-methyl-1-butanol, 22- 2-methyl-1-propanol, 23- Ethyl lactate, 24- 1-pentanol, 25- Octanal, 26- 1-hexanol, 27- 2-nonanone, 28- Verbenone, 29- Ethyl octanoate, 30 Furfural, 31- Decyl aldehyde, 32- Furfuryl acetate, 33- Isobutyric acid, 34- Diethyl malonate, 35- Furfuryl propionate, 36- 1,2-propanediol, 37- Phenyl acetate, 38- Butyric acid, 39- Propyl butyrate, 40- Furfuryl alcohol, 41- Diethylsuccinate, 42- b-Citronellol, 43- Guaiacol, 44- Decanoic acid, 45- Propionic acid, 46- a-Terpeniol, 47- Geraniol, 48 Trans-3-hexen-1-ol.

3.5.Sensory analysis

The temporal dominance of sensations consists in assessing iteratively at each specific time until the sensations end, which sensation is dominant and in scoring its intensity (Labbe, Schlich, Pineau, Gilbert & Martin, 2009). Figures 6 and 7 show the TDS curves for the washed and non-washed coffee fermented via dry process with yeasts inoculated as cultures starters. The samples from the control treatment of washed coffees (Figure 6A) showed an early perception as caramel sensation and after 5 s the aroma was dominated by chocolate and fruit, and finally after 20 s it was possible to feel a strong bitter flavor. The samples from the treatments with starter cultures showed sensory differences when compared with the control treatment, except for the ones inoculated with UFLA YCN731 (Figure 6 B-E).

Each inoculated sample showed a strongly dominant sensation and different between them, for example, when samples of treatment with UFLA YCN448 showed a strong feeling of caramel 5s, UFLA YCN724 showed a strong acidic sensation between 5 and 10s, UFLA YCN727 showed a strong fruity feeling 15s after ingestion. All grains tested and inoculated with *C. parapsilosis* UFLA YCN448 (Figure 6B) had the

highest rate of dominance (near 1) for the sensation of caramel and, at the end, (about 20s) the feeling was predominantly herbaceous. Thus, it was possible to observe that in all washed coffee treatments with selected yeasts inoculum showed greater sensations and differences compared to control, suggesting an increase in flavor and therefore greater acceptance of tasters.

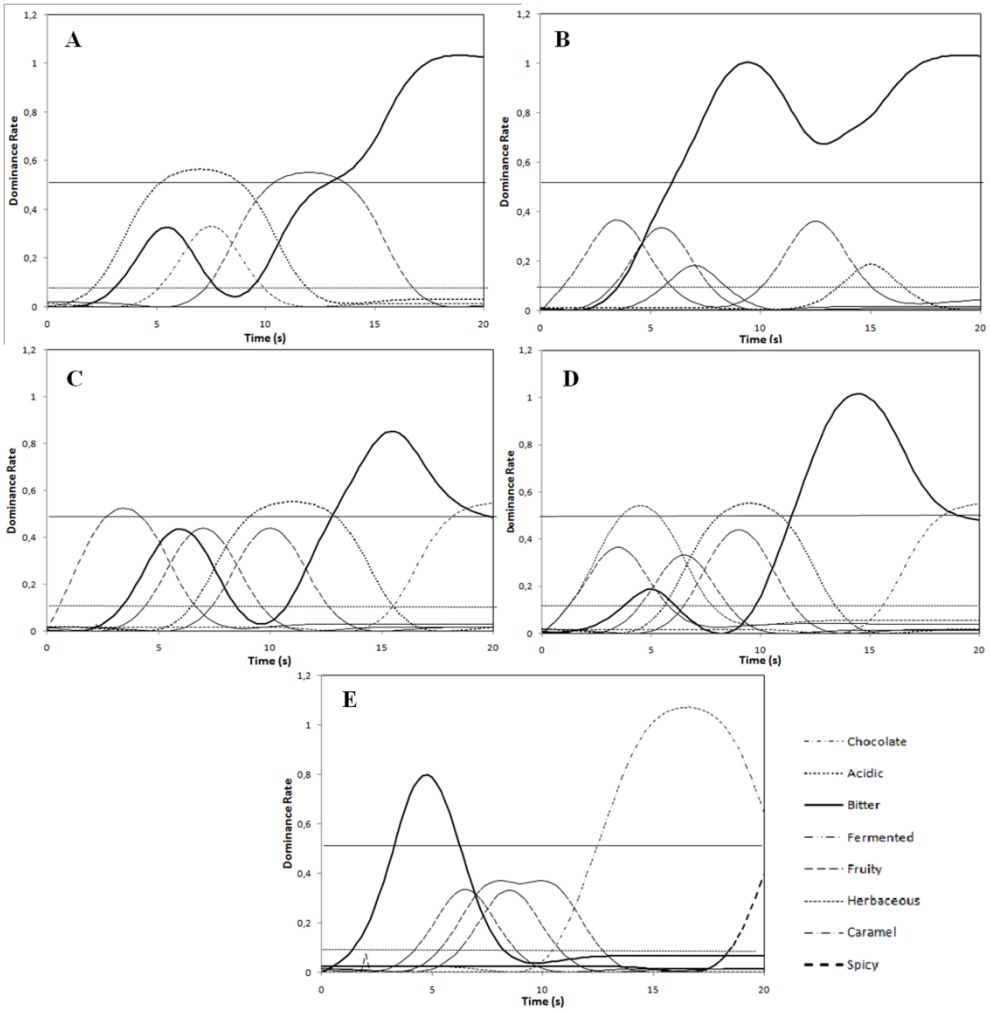

Figure 6. TDS graphical representation for the washed coffee samples after the roasting from beans inoculated with yeasts strains (Control (A); *C. parapsilosis* UFLA YCN448 (B), *S. cerevisiae* UFLA YCN724 (C), *S. cerevisiae* UFLA YCN727 (D), *P. guilliermondii* UFLA YCN731 (E)). Significance level (⁻) and chance level (⋯).

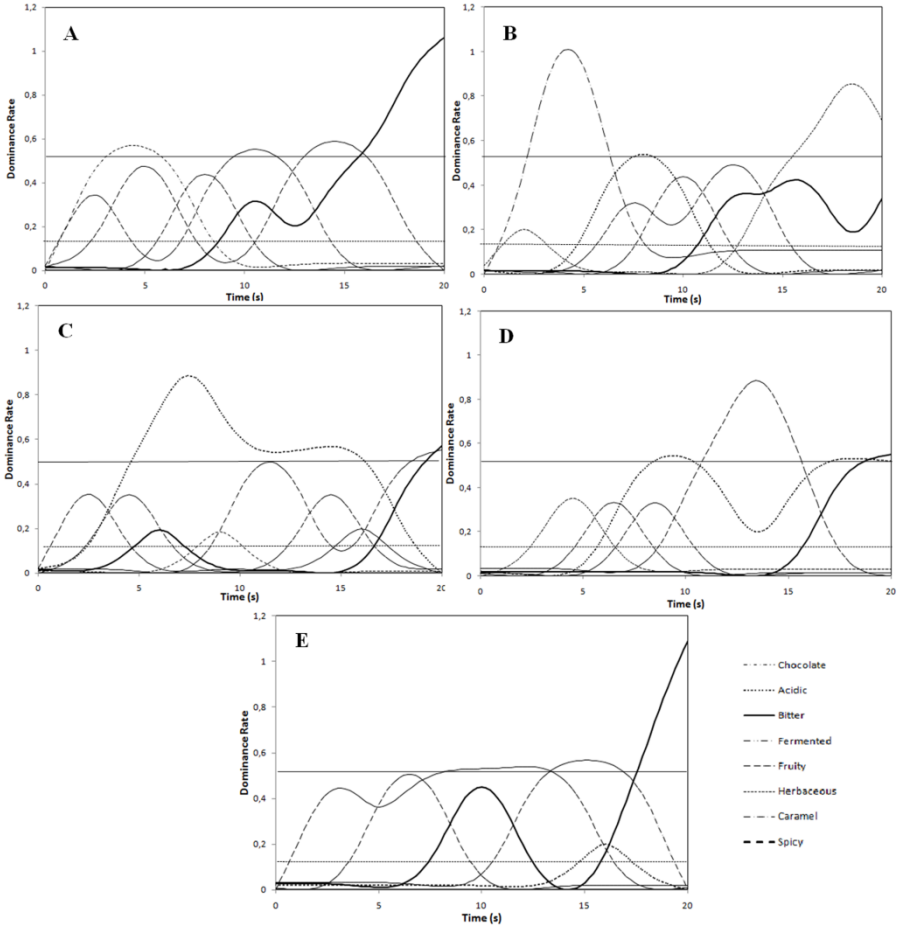

Figure 7. TDS graphical representation for the non-washed coffee samples after the roasting from beans inoculated with yeasts strains (Control (A); *C. parapsilosis* UFLA YCN448 (B), *S. cerevisiae* UFLA YCN724 (C), *S. cerevisiae* UFLA YCN727 (D), *P. guilliermondii* UFLA YCN731 (E)). Significance level (⁻) and chance level (···).

The control samples of non-washed coffee (Figure 7A) showed bitterness as the first perceived aroma / flavor, followed by the acidity and chocolate (between 5-10s) and finally after 20s a strong bitterness flavor. Bitterness feeling in late 20s was also prevalent in the washed coffee without yeasts inoculation. The grains inoculated with UFLA YCN448 (Figure 7B) showed initial sensation of caramel (however weak) when there was a predominant and strong feeling of bitterness after 10s. Grains inoculated with UFLA YCN724 (Figure 7C) showed that the initial sensation was perceived as fermented with predominance after 20s of the bitterness feeling. Grains inoculated with UFLA YCN727 (Figure 7D) showed an initial sensation of caramel (weak) with final predominance (20s) of a bitterness feeling. Coffee beans inoculated with UFLA YCN731 (Figure 7E) showed an initial bitterness sensation but with an intense chocolate sensation after 20s. Thus, washed coffees inoculated with yeasts as starter culture may be used in blends to achieve different flavor.

Non-washed coffee grains inoculated with yeast presented more pleasant sensations than the experiments where the coffee was washed before inoculation, except for inoculation with the UFLA YCN731 strain.

These results suggested that washing the coffee fruits before the fermentation process influenced the final quality of the product. The non-washed coffee also had the lowest scores (73.25 on average) in the cup proof compared with washed coffee samples (77.18 average rate).

Thus, it was possible to conclude that it is possible to use selected yeasts for the fermentation of coffee natural or dry processing. The inoculated yeasts persisted during the entire fermentation and resulted in a beverage with a distinctive flavor (caramel and fruity) and good sensory quality. Further research should focus on choosing selected strains for inoculation in washed coffee fermented by dry method in order to increase the pleasant sensations obtained for non-washed coffee. The use of starter cultures in coffee fermentation is an economically viable alternative to obtain a differentiated coffee, adding value to the product and standardizing the dry process.

Acknowledgments

The authors thank the following Brazilian agencies: Conselho Nacional de Desenvolvimento Científico e Tecnológico of Brasil (CNPQ); Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG);

and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for scholarships, and the coffee producers from Minas Gerais State, Brazil for the collecting of samples.

References

Bertrand, B., Boulanger, R., Dussert, S., Ribeyre, F., Berthiot, L., Descroix, F., & Joët, T. (2012). Climatic factors directly impact the volatile organic compound fingerprint in green Arabica coffee bean as well as coffee beverage quality. *Food Chemistry 135*(4), 2575-2583.

Buffo, R. A., & Cardelli-freire, C. (2004). Coffee flavour: an overview. *Flavour and Fragrance Journal 19* (2), 99-104.

Cantergiani, E., Brevard, H., Krebs, Y., Feria-Morales, A., Amado, R., & Yeretzian, C., (2001). Characterisation of the aroma of green Mexican coffee and identification of mouldy/earthy defect. *European Food Research and Technology 212*, 648-657.

Cocolin, L., Bisson, L. F., & Mills, D. A. (2000). Direct profiling of the yeast dynamics in wine fermentations. *FEMS Microbiology Letters 189*, 81-87.

Czerny, M., &Grosch, W., (2000). Potent odorants of raw Arabica coffee. Their changes during roasting. *Journal Agriculture and Food Chemistry 48*, 868-872.

Duarte, W. F., Dias, D. R., Oliveira, J. M.,Teixeira, J. A., Silva, J. B. A., & Schwan, R. F. (2010). Characterization of different fruit wines made from cacao, cupuassu, gabiroba, jaboticaba and umbu. *LWT - Food Science and Technology 43*(10), 1564-1572

Esquivel, P., & Jiménez, V. M. (2012). Functional properties of coffee and coffee by-products. *Food Research International 46*(2), 488-495.

Farah, A., Monteiro, A.C., Calado, V., Franca, A.S., &Trugo, L.C. (2006). Correlation between cup quality and chemical attributes of Brazilian coffee. *Food Chemistry 98,* 373–380.

Fuster, M.D., Mitchell, A.E., Ochi, H., &Shibamoto, T., (2000). Antioxidative activities of heterocyclic compounds formed in brewed coffee. *Journal of Agricultural and Food Chemistry 48,*5600-5603.

Guyot, B., Cros, E.,& Vincent, J.C., (1982). Caractérisation et identification des composés de la fraction volatile d'un café vert Arabica sain et d'un café vert arabica puant. *Café, Cacao, Thé (France) 26* (4), 279-289.

Joët, T., Laffargue, A., Descroix, F., Doulbeau, S., Bertrand, B., Kochko, A. D., & Dussert, S. (2010). Influence of environmental factors, wet processing and their interactions on the biochemical composition of green Arabica coffee beans. *Food Chemistry1 18*(3), 693-701.

Knopp, S., Bytof, G., & Selmar, D. (2005). Influence of processing on the content of sugars in green Arabica coffee beans. *European Food Research and Technology 223*(2), 195-201

Labbe, D., Schlich, P., Pineau, N., Gilbert, F.,& Martin, N., (2009). Temporal dominance of sensations and sensory profiling: a comparative study. *Food Quality and Preference 20,* 216-221.

Leroy, T., Ribeyre, F., Bertrand, B., Charmetant, P., Dufour, M., Montagnon, C., Marraccini, P. & Pot, D., (2006). Genetics of coffee quality. *Braz. J. Plant Physiol.18,* 229-242.

Lopez, C.I., Bautista, E., Moreno, E., & Dentan, E., 1989. Factors related to the formation of "overfermented coffee beans" during the wet processing method and storage of coffee. *ASIC*, 13 Colloque, Paipa, 1989. ISBN 2-900212-12-X. p. 373-384.

Masoud, W., Cesar, L. B., Jespersen, L., & Jakobsen, M. (2004). Yeast involved in fermentation of *Coffea arabica* in East Africa determined by genotyping and by direct denaturating gradient gel electrophoresis. *Yeast 21*, 549-556.

Masoud, W., Poll, L.,& Jakobsen, M., (2005). Influence of volatile compounds produced by yeasts predominant during processing of *Coffea arabica* in East Africa on growth and ochratoxin A (OTA) production by *Aspergillus ochraceus. Yeast 22*, 1133–1142.

Nübel, U., Garcia-Pichel,F., Kuhl, M., & Muyzer, G., (1999). Quantifying microbial diversity: morphotypes, 16 SrDNA genes and carotenoids of oxygenic phototrophsin microbial mats. *Applies Environmental Microbiology 65*, 422-430.

Nunes, C. A., & Pinheiro, A. C. M., 2012. Sensomarker Free user-friendly interface for sensory analysis, 2012. Available from: www.ufla.br/sensomaker. Accessed at: jun. 2013

Ovreas, L., Forney, L., & Daae, F. L. (1997). Distribution of bacterioplankton in meromictic lake Sælenvannet, as determined by denaturing gradient gel electrophoresis of PCR-Amplified gene fragments coding for 16S rRNA. *Applied and Environmental Microbiology 63*(9), 3367–3373.

Petisca, C., Pérez-Palacios, T., Farah, A., Pinho, O., & Ferreira, I. M. P. L. V. O. (2013). Furans and other volatile compounds in ground roasted and espresso coffee using headspace solid-phase microextraction: Effect of roasting speed. *Food and Bioproducts Processing 91*(3), 233-241.

Pineau, N., Schlich, P., Cordelle, S., Mathonnière, C., Issanchou, S., Imbert, A., & Rogeaux, M., et al. (2009). Temporal Dominance of Sensations: Construction of the TDS curves and comparison with time–intensity. *Food Quality and Preference 20*(6), 450-455.

Ramos, C.L., Almeida, E.G. de, Pereira, G.V. de M., Cardoso, P.G., Dias, E.S.D.,& Schwan, R.F., (2010). Determination of dynamic characteristics of microbiota in a fermented beverage produced by Brazilian Amerindians using culture-dependent and culture-independent methods. *InternationalJournal of Food Microbiology 140* (2-3), 225-231.

Sanz, C., Maeztu, L., Zapelena, M. J., Bello, J.,& Cid, C., (2002). Profiles of volatile compounds and sensory analysis of three blends of coffee: influence of different proportions of Arabica and Robusta and influence of roasting coffee with sugar. *Journal of the Science of Food and Agriculture 82*, 840-847.

Schwan, R. F., Silva C. F., Batista L. R., (2012). Coffee fermentation. In: Hui, Y. H . (Ed), Handbook of plant-based fermented food and beverage technology(pp 677–690). CRC Press, Boca Raton

Silva, C F, Schwan, R. F., Sousa Dias, E. S., & Wheals, A. E. (2000). Microbial diversity during maturation and natural processing of coffee cherries of *Coffea arabica* in Brazil. *International Journal of Food Microbiology 60*(2-3), 251-60.

Silva, C. F., Batista, L. R., Abreu, L. M., Dias, E. S., & Schwan, R. F. (2008). Succession of bacterial and fungal communities during natural coffee (*Coffea arabica*) fermentation. *Food Microbiology 25*(8), 951-957.

Silva, Cristina Ferreira, Vilela, D. M., Cordeiro, C. S., Duarte, W. F., Dias, D. R., & Schwan, R. F. (2013). Evaluation of a potential starter culture for enhance quality of coffee fermentation. *World Journal of Microbiology & Biotechnology 29*(2), 235-47.

Toci, A.T., &Farah, A., (2008). Volatile compounds as potential defective coffee beans' markers. *Food Chemistry 108* 1133-1141.

Vilela, D. M., Pereira, G. V. D. M., Silva, C. F., Batista, L. R., & Schwan, R. F. (2010). Molecular ecology and polyphasic characterization of the microbiota associated with semi-dry processed coffee (*Coffea arabica L*.). *Food Microbiology*, *27*(8), 1128-35.

SCAA- Specialty Coffee Association of America. 2012. Available in: <http://www.scaa.org> Accessed at: out. 2012.

Yanagimoto, K., Lee, K-G.,Ochi, H., &Shibamoto, T. (2004). Antioxidative activity of heterocyclic compounds found in coffee volatiles produced by Maillard reaction. *Journal of Agricultural and Food Chemistry 52*, 592-596.

Yanagimoto, K., Lee, K-G.,Ochi, H., &Shibamoto, T. (2002).Antioxidative activities of fractions obtained from brewed coffee.*Journal ofAgricultural and Food Chemistry 50*,5480-5484.

Yeretzian, C., Jordan, A., Badoud, R., &Lindinger, W., (2002). From the green bean to the cup of the coffee: investigating coffee roasting by on-line monitoring of volatiles. *European Food Research and Technology 214*, 92-104.

Wakeling, I. N., &Macfie, H. J. H., (1995). Designing consumer trials balanced for first and higher orders of carry-over effect when only a subset of k samples from t may be tested. *Food Quality and Preference 6*, 299-308.

ARTIGO 2 Inoculation of starter cultures in a semi-dry coffee (*Coffea arabica*) fermentation process

Suzana Reis Evangelista[1], Maria Gabriela da Cruz Pedrozo Miguel[2], Cecília de Souza Cordeiro[2], Cristina Ferreira Silva[2], Ana Carla Marques Pinheiro[1], Rosane Freitas Schwan[2*]

[1]Food Sciences Department, Federal University of Lavras, CEP 37200-000, Lavras, MG, Brazil

[2]Biology Department, Federal University of Lavras, CEP 37200-000, Lavras, MG, Brazil

*Corresponding author

x (+55) 35 3829 1100; e-mail: rschwan@dbi.ufla.br]

Running title: Use of starter cultures in semi-dry coffee fermentation

Artigo científico submetido para o periódico: *Food Microbiology*.

ABSTRACT

The aim of this study was to evaluate the use of yeasts as starter cultures in coffee semi-dry processing. Arabica coffee was inoculated with one of the following starter cultures: *Saccharomyces cerevisiae* UFLA YCN727, *Saccharomyces cerevisiae* UFLA YCN724, *Candida parapsilosis* UFLA YCN448 and *Pichia guilliermondii* UFLA YCN731. The control was not inoculated with a starter culture. Denaturing gradient gel electrophoresis (DGGE) was used to assess the microbial population, and organic acids and volatile compounds were quantified by HPLC and HS-SPME/GC, respectively. Sensory analysis were evaluated using the Temporal Dominance of Sensations (TDS). DGGE analysis showed that the inoculated yeasts were present throughout the fermentation. Other yeast species were also detected, including *Debaryomyces hansenii*, *Cystofilobasidium ferigula* and *Trichosporon cavernicola*. The bacterial population was diverse and was composed of the following genera: *Weissella*, *Leuconostoc*, *Gluconobacter*, *Pseudomonas*, *Pantoea*, *Erwinia* and *Klebsiella*. Butyric and propionic acids, were not detected in any treatment A total of 47 different volatiles compounds have been identified. The coffee inoculated with yeast had a caramel flavor that was

not detected in the control, as assessed by TDS. The use of starter cultures during coffee fermentation is an interesting alternative for obtaining a beverage quality with distinctive flavor.

Keywords: semi-dry processing, yeast, metabolites, PCR-DGGE.

1. Introduction

Brazil is the largest coffee producer in the world (4.2 Mtons), followed by Paraguay, Venezuela, Colombia, Indonesia, Ethiopia, India, Mexico and 40 other countries. Two coffee species dominate the world market: *Coffea arabica* (arabica) and *C. canephora* (robusta). Arabica and robusta coffees account for 76.4% and 23.6% of world production, respectively (Coltro et al., 2006). Coffee is one of the most popular and appreciated non-alcoholic beverages, and its consumption is global. Coffee fruits are processed by three different methods – dry, wet and semi-dry (Schwan et al., 2012). The most simple and rustic process is dry processing, which is used to ferment and sundry coffee on platforms and/or cement. In wet processing, the pulp and/or mucilage are

mechanically removed, and the grains are fermented in tanks with a large volume of water. The semi-dry process is a variation of the wet process, in which coffee fruits are depulped, but the fermentation process occurs directly under the sun on a platform (Vilela et al., 2010).

During coffee processing, a large microbial diversity is present. Yeast, filamentous fungi and Gram-negative and Gram-positive bacteria are present in high numbers during the different stages of fermentation (Schwan and Wheals, 2003). The population of bacteria is greater than the yeast population at the beginning of fermentation, independent of the process. The most common bacterial genera present during coffee fermentation are *Lactobacillus*, *Bacillus*, *Arthrobacter*, *Acinetobacter*, *Klebsiella* and *Weissella*. Yeasts tend to increase during fermentation/drying and can reach greater values than the bacterial population. *Saccharomyces*, *Pichia*, *Candida*, *Rhodotorula*, *Hanseniaspora* and *Kluyveromyces* are the most common yeast genera found. The filamentous fungi are usually found in smaller quantities and are of note during drying and in storage, in particular. *Aspergillus*, *Penicillium*, and *Fusarium* are the main fungi genera present (Masoud et al., 2004; Silva et al., 2008; Vilela et al., 2010).

The use of starter cultures improves the quality of fermented foods by providing better fermentation control and predictability of the final product. These microbial cultures are selected as single or multiple strains (Holzapfel, 2002). The selection of specific microorganisms for starter cultures to be used during coffee fermentation are important to prevent the growth of filamentous fungi, especially producers of ochratoxin A (OTA) (Massawe and Lifa, 2010); to enhance the quality of the fermentation process and to improve the sensory quality of the final beverage. Pectinolytic yeasts secreting these enzymes in suitable amounts may be added during coffee fermentation to accelerate the processing and enhance the coffee quality (Schwan & Wheals, 2003).

In studies coffee performed by Silva et al. (2013) showed that the yeasts *S. cerevisiae* UFLACN727, *P. guilliermondii* UFLACN731 and *C. parapsilosis* UFLACN448 have good potential to be used as starter cultures for coffee fermentation. These isolates stood out, among others, due to their enzymes secretion that hydrolyzes the coffee mucilage accelerating and improving the quality of the fermentation process. Besides, the authors showed that the production of organic acids and

volatile compounds by those yeasts might contribute to the final quality of the beverage.

A factor that greatly contributes to the high acceptability of coffee by consumers is the aroma, which involves more than 800 volatile compounds. Its complex composition depends on the botanical variety of coffee and the methods for processing, grinding, packaging, roasting, and extraction (Charles-Bernard et al., 2005). Gas chromatography with solid phase microextraction (SPME-GC) is an excellent method for analyzing and separating volatile fractions and is one of the most important for evaluating metabolic compounds (Gonzales-Rios et al., 2007).

The objectives of this work were to evaluate the effects of four yeasts as starter cultures during semi-dry coffee processing through microbiological analysis by denaturing gradient gel electrophoresis (DGGE), biochemical analysis by high performance liquid chromatography (HPLC) and headspace solid-phasemicroextraction/gas chromatography (HS-SPME/GC) and sensory analysis by Temporal Dominance of Sensations (TDS).

2. Materials and Methods

2.1. Sampling

Coffee cherries (*Coffea arabica* L. var. Acaiá) were manually harvested at the mature stage (red cherries) from a farm 750 - 800 m above sea level situated in Lavras in the state of Minas Gerais, Brazil. The beans were processed using the semi-dry method, where the cherries were mechanically depulped in a horizontal machine (model BDSV-04; Pinhalense, São Paulo, Brazil).

2.2. Microorganisms used as starters cultures

The yeast isolates used as starters cultures in coffee fermentation were *Saccharomyces cerevisiae* UFLA YCN727, *Saccharomyces cerevisiae* UFLA YCN724, *Candida parapsilosis* UFLA YCN448 and *Pichia guilliermondii* UFLA YCN731. The yeast isolates were obtained from the Culture Collection of the Laboratory of Microbial Physiology at DBI/UFLA, Lavras, Minas Gerais State, Brazil. The microorganisms had been previously isolated from coffee fruit (*Coffea arabica* L. var. Acaiá) during dry and semi-dry fermentative processes (Silva et al., 2000; Vilela

et al., 2010), as well as their potential for use as starters cultures in coffee fermentation (Silva et al., 2013).

2.2.1. Preparation of Inoculum

Isolates were stored at –80 ºC and were reactivated in YEPG tubes containing 5 mL of liquid medium [ing L^{-1}: glucose 20 (Merck), yeast extract 10 (Merck), peptone soy 10 (Himedia) and agar 20 (Merck), pH 3.5]. The cultures were incubated at28 ºCfor48 h, and then transferred to50 mL of YEPG and incubated at 28 °C, 150 rpm for 24 h. The yeast cells were transferred to larger volumes of YEPG until a concentration of 10^7cells/mL was reached.

2.2.2. Inoculation of starter cultures

The pulped coffee were spread (600 g) on a tray and each treatment was inoculated separately with one of the following yeasts: *S. cerevisiae* UFLA YCN727, *S. cerevisiae* UFLA YCN724, *C. parapsilosis* UFLA YCN448 and *P. guilliermondii* UFLA YCN731. The control treatment was not inoculated with any yeast strain. Coffee cherries were fermented and sun dried until they reached 11-12% of moisture. During

fermentation and drying, samples were collected aseptically placed in sterile plastic bags, and transferred to the Microbiology Laboratory of the Federal University of Lavras for microbiological and chemical analyses.

2.3. Enumeration of microorganisms

Each sample (2 g of fruits) was added to a bottle containing 18 mL of saline-peptone water [ing L^{-1}: 1bacteriological peptone (Himedia)]. After mixing for 20 min at 150 rpm in an orbital shaker, ten-fold dilutions were prepared. Microorganisms were counted using two different culture media: PCA [ing L^{-1}: tryptone5 (Himedia), yeast extract 2.5 (Merck), glucose 1 (Merck), agar 15 (Merck)] was used as a general medium for the viable bacteria population and YEPG [ing L^{-1}: glucose20 (Merck), yeast extract 10 (Merck), peptone soy 10(Himedia) and agar20 (Merck), pH 3.5] was used for yeasts. Following inoculation, the plates were incubated at 28 °C for 48 h.

2.4. Microbial community analysis through PCR-DGGE

2.4.1. Total DNA extraction and PCR analysis

Three grams of sample was mixed with 5 mL of Milli-Q water for 10 min, followed by centrifugation at 100×g for 10 min at 4 °C. The pellet was used for DNA extraction. Total DNA was extracted from samples at three times of fermentation [initial (0 h), intermediate (312 h) and end (720 h)] using the protocol "DNA Purification from Tissues" [QIAamp DNA Mini Kit (Qiagen, Hilden, Germany)] in accordance with the manufacturer's instructions. The DNA from the bacterial community was amplified with the primers 338fgc (5′ CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG 3′) (the GC clamp is underlined) and 518r (5′ ATT ACC GCG GCT GCT GG 3′), which span the V3 region of the 16S rRNA gene (Ovreas et al., 1997). A fragment of the D1-region of the 26S rRNA gene was amplified with the eukaryotic universal primers NL1GC (5′ CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA TAT CAA TAA GCG GAG GAA AAG 3′) (the GC clamp is underlined) and LS2 (5′ ATT CCC AAA CAA CTC GAC TC 3′), which amplified a fragment of approximately 250 bp (Cocolin et al., 2000). PCR was carried out in a

final reaction volume of 25 µL containing 0.625 U Taq DNA polymerase (Promega, Milan, Italy), 2.5 µL 10× buffer, 0.1 mM dNTPs, 0.2 mM of each primer, 1.5 mM $MgCl_2$ and 1 µL of extracted DNA. The amplification was performed according to Ramos et al., 2010. Aliquots (3 µL) of the amplification products were analyzed by electrophoresis on 1% agarose gels before they were used for DGGE.

2.4.2. PCR-DGGE analysis

The PCR products were separated in polyacrylamide gels [8% (w/v) acrylamide: bisacrylamide (37.5:1)] in 1× TAE buffer with a DCode system apparatus (BioRad Universal Dcode Mutation Detection System, Richmond, CA, USA). Denaturation gradients were used that varied from 15 to 55% for the bacterial products (100% corresponded to 7 M of urea and 40% [v/v] of formamide) and 20 to 60% for the yeast products. Electrophoresis was conducted at a constant voltage of 130 V for 6 h (bacteria and yeast) and at a constant temperature of 60 °C. Following electrophoresis, the gels were stained with SYBR-Green I (Molecular Probes, Eugene, UK) (1:10.000 v/v) for 30 min. The images

were visualized and photographed using a transilluminator LPixImage (LTB 20 X 20 HE, LPix®, Brazil).

2.4.3. DGGE band sequencing

Selected bands from the PCR–DGGE gels were excised with a sterile blade and placed in 50 μL of sterile Milli-Q water at 4 °C overnight to allow the DNA to diffuse out of the polyacrylamide matrix. The samples were then amplified using the primers 338fgc and 518r for bacteria and NL1 and LS2 for yeast. The DNA fragments were purified with a QIAEX II gel extraction kit (Qiagen, Chatsworth, CA, USA), and the PCR products were sequenced at the University UNESP (Jaboticabal, São Paulo, Brazil) on the automated sequencer ABI 3730 XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA). The sequences were compared with those available in the GenBank database using the BLAST algorithm (National Center for Biotechnology Information, Maryland, USA).

2.5. Chromatographic analysis of the coffee

2.5.1. Characterization of organic acids by HPLC

Organic acids (malic, lactic, acetic, butyric, propionic, citric, oxalic, succinic and tartaric acid) were analyzed using a high-performance liquid chromatography system (Shimadzu Corp., Japan) with a UV detector at 210 nm. Three grams of sample was mixed with 5 mL of Milli-Q water for 10 min, and the fluids were centrifuged twice at 100×g for 10 min at 4 °C (Silva et al., 2008). The samples were microfiltered through a 0.2 µm cellulose acetate filter and directly injected (20 µl) onto the chromatography column. A Shimpack SCR-101H (7.9 mm × 30 cm) column operated at 50 °C was used to achieve chromatographic separation of water-soluble acids that were eluted with 100 mM of perchloric acid at a flow rate of 0.6 mL/min. The acids were detected by UV absorbance (210 nm) and were identified by comparison with retention times of authentic standards. 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)] analyzed using the same conditions as for the samples (Duarte et al., 2010).

2.5.2. Characterization of volatile compounds by HS-SPME/GC

2.5.2.1. Extraction of volatiles by headspace-SPME

Coffee samples were macerated using nitrogen for headspace analysis. A divinylbenzene / carboxen / polydimethylsiloxane (DVB / CAR / PDMS) 50/30 µm SPME fiber (Supelco Co., Bellefonte, PA, USA) was used to extract volatile constituents from the coffee headspace. Two grams of coffee were placed in a 15 mL hermetically sealed flask and heated for 10 min at 60 °C to reach sample headspace equilibrium. Then, volatile compounds were extracted by placing the SPME fiber in the headspace for 30 min at 60 °C. For compound desorption, the fiber was placed in the GC injection port heated to 230 °C for 5 min (Gonzales-Rios et al., 2007).

2.5.2.2. HS-SPME/GC analysis

Analysis was performed using a gas chromatograph (GC), Shimadzu model 17A equipped with an FID (flame ionization detector) and a capillary column of silica DB wax (30 m × 0.25 mm i.d. × 0.25 μm) (J&W Scientific, Folsom, Calif., U.S.A.). The oven temperature was maintained at 50 °C for 5 min. The temperature was then raised to 190 °C in increments of 3 °C/min and then maintained at 190 °C for 10 min. Injector and detector temperatures were kept at 230 and 240 °C, respectively. The carrier gas (N_2) was maintained at a flow rate of 1.2 mL/min. Volatile compounds were identified by comparing the retention times of the compounds with those of standard compounds injected under the same conditions (Duarte et al., 2010).

The volatile compounds from each headspace analysis were defined by integrating the peak areas of all the identified compounds. The relative percentages of individual compounds were calculated from the total contents of volatiles on the chromatograms (Petisca et al., 2013).

2.6. Analysis of sensory characteristics

The samples were prepared according to the Specialty Coffee Association of America (SCAA, 2013). The coffee was roasted in a laboratory roaster (Probatino, Leogap model, Brazil) with a capacity of 150 g and was then ground in an electric mill (Pinhalense ML-1, Brazil). A panel of three trained coffee tasters with Q-Grader Coffee Certificates evaluated the samples in this study. Two methodologies were applied to evaluate coffee sensorially. The first sensorial evaluation was conducted according to SCAA standards (SCAA, 2013), assessing ten sensorial attributes: fragrance, flavor, aftertaste, acidity, body, uniformity, balance, sweetness, cleanliness and score.

The Temporal Dominance of Sensations (TDS) analysis (Pineau et al., 2009) was used to identify the most relevant attributes for describing the temporal evolution of sensations for each sample by a panel of trained coffee experts. The attributes selected by the panel were as follows: chocolate, acidic, bitter, fermented, fruity, herbaceous, caramel, and spices. The total duration of the test was 20 s. The panelist imbibed the coffee, moved it around in their mouths for 3 s and then swallowed it. The evaluation continued until no sensation was perceived or at 20 s. The act

of swallowing the sample facilitated the temporal sensory perception of the product according to the panelist's reports. Samples coded with three digits were submitted in balanced order (Wakeling and MacFie, 1995) and evaluated in three replicates. The data were recorded using the SENSOMAKER Software (Nunes and Pinheiro, 2012) and plotted as TDS curves showing the percentage of subjects who selected the attribute as dominant at a specific time; i.e., the dominance rate (Pineau et al., 2009).

2.7. Statistical analysis

The volatile compound data were statistically analyzed (principal component analysis, PCA) using SENSOMAKER Software (Nunes and Pinheiro, 2012). An $m \times n$ matrix was built with the relative areas of the n identified chromatographic picks for the m samples for volatile compounds.

3. RESULTS

3.1. Microbiological analyses

The microbial populations of bacteria and yeast during the fermentation of the four treatments and the control (without inoculum**)** are shown in Fig. 1.Bacterial numbers were higher than yeast numbers during the fermentation process (Fig. 1A). The control had lower bacterial and yeast populations compared to the other treatments, reaching maximum values of 7.5 and 6.4 log CFU/g, respectively. The coffee samples inoculated with yeast had bacterial populations of approximately 8 log CFU/g after the 7^{th} day (Fig. 1A).

The yeast population was lower in the control during the entire fermentation process, reaching maximum values of 6 log CFU/g. The treatment inoculated with UFLA YCN448 had the largest population of yeast, ranging between 6.18 and 7.77 log CFU/g. The treatments inoculated with *P. guilliermondii* UFLA YCN731 and *S. cerevisiae* UFLA YCN724 hadsimilar yeast counts (approximately 7.61 log CFU/g).

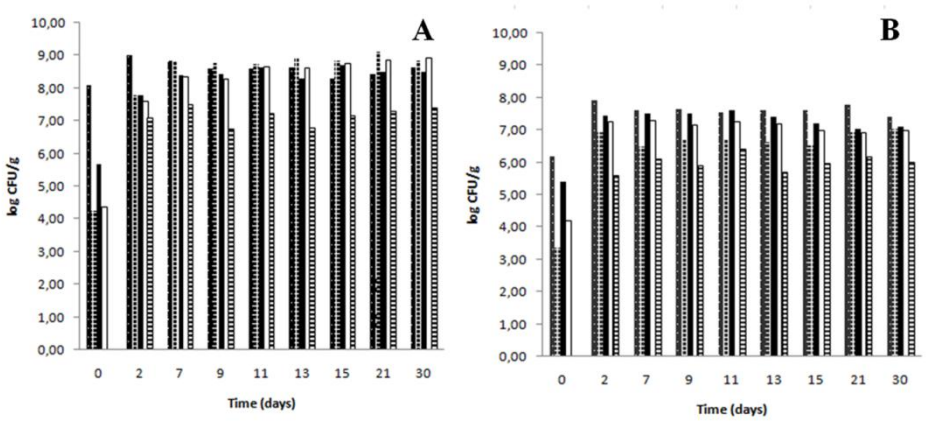

Fig. 1 Population of bacteria (A) and yeast (B) in coffee inoculated with yeasts. (▩) *Candida parapsilosis* UFLA YCN448, (▩) *Saccharomyces cerevisiae* UFLA YCN724, (■) *Pichia guilliermondii* UFLA YCN731, (□) *Saccharomyces cerevisiae* UFLA YCN727, and (▬) control without inoculum.

3.2. PCR-DGGE analysis of yeast and bacterial diversity

The diversity of the microbial community in coffee semi-dry processing using yeasts as starter cultures were evaluated using PCR-DGGE (Fig. 2 and 3). The inoculated yeasts dominated and were present during the entire fermentation, as shown in Fig. 2. The yeast populations were diverse, composed of 7 distinct species belonging to 7 genera (Fig. 2 and Table 1), including the yeast used as starters cultures.

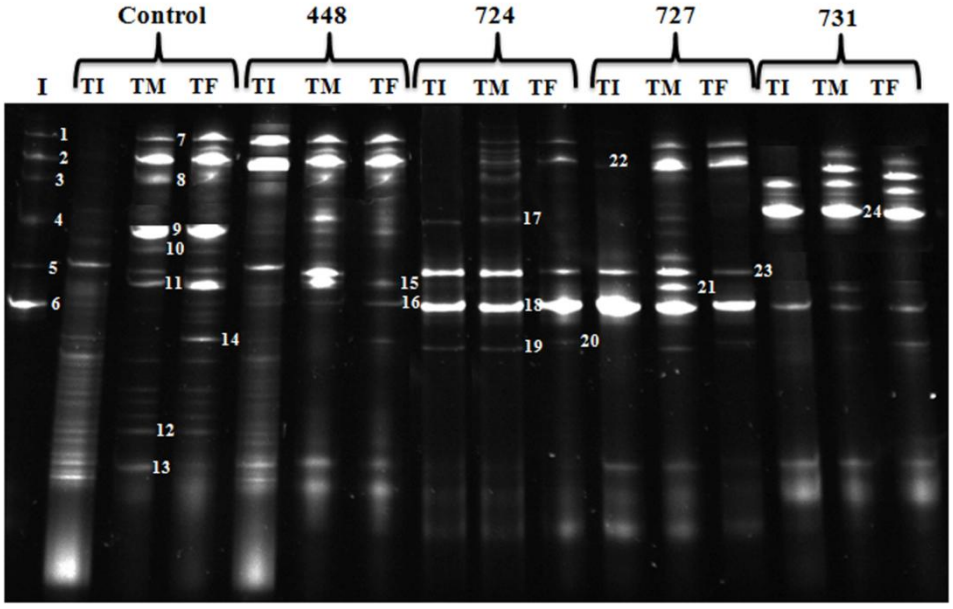

Fig. 2PCR-DGGE patterns of the yeast communities present during semi-dry coffee processing. TI = 0 h, TM = 312 h and TF = 720 h fermentation. I = mix starter culture used in fermentation. Coffee without inoculums (control) and inoculated with *Candida parapsilosis* UFLA YCN448 (448), *Saccharomyces cerevisiae* UFLA YCN724 (724), *S. cerevisiae* UFLA YCN727 (727) and *Pichia guilliermondii* UFLA YCN731 (731).

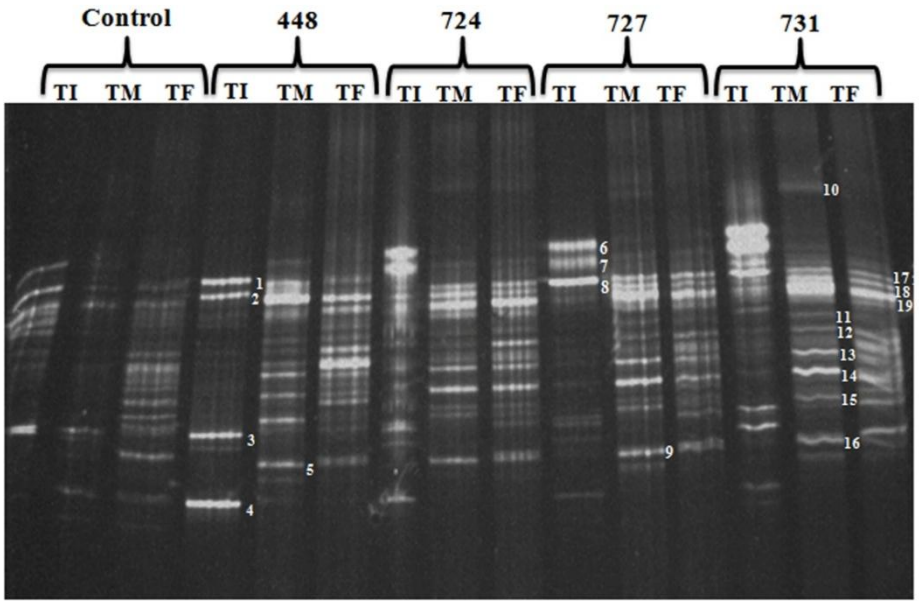

Fig. 3PCR-DGGE patterns of the bacterial communities present during semi-dry coffee processing. TI = 0 h, TM = 312 h and TF = 720 h fermentation. Coffee without inoculums (control) and inoculated with *Candida parapsilosis* UFLA YCN448, *Saccharomyces cerevisiae* UFLA YCN724, *S. cerevisiae* UFLA YCN727 and *Pichia guilliermondii* UFLA YCN731.

Table 1 Species identified by PCR-DGGE using universal primers for yeast and bacteria.

Bands[a]	**Access number**	**Similarity (%)**	**Eukaryote**
1, 2, 7, 22	FJ 009291	98	*Candida parapsilosis*
3, 4, 8, 9, 17, 24	EF 197951	99	*Pichia guilliermondii*
5, 6, 16, 18, 23	EU 649672	100	*Saccharomyces cerevisiae*
10	FJ 475230	100	*Debaryomyces hansenii*
11, 15, 21	FR 819697	100	*Cystofilobasidium ferigula*
12	--	--	Unidentified
13	--	--	Unidentified
14, 20	JQ 417238	99	*Mitchella repens*
19	AB 180195	100	*Trichosporon cavernícola*
			Prokaryote
1	GU 470977	99	*Weissella soli*
2, 8	GU 458344	99	*Leuconostoc mesenteroides*
3, 4	NR 074252	100	*Gluconobacter oxydans*
5, 9	FJ 609991	98	Uncultured *Enterobacter* sp.
6, 7, 19	GU 301189	98	Uncultured bacterium
10	JX 514422	99	*Pseudomonas aeruginosa*
11	EU 741017	99	*Pantoea* sp.
12	JX 865456	100	*Pantoea eucrina*
13	JQ 353805	100	*Erwinia toletana*
14	KC 153120	99	*Erwinia tasmaniensis*
15	FR 821664	99	*Klebsiella oxytoca*
16, 18	--	--	Unidentified
17	FJ 481992	98	*Morganella morganii*

As can be seen in Fig. 2, the species *C. parapsilosis*, used as the inoculum for the treatment UFLA YCN448, was also detected in the

control and in treatments inoculated with UFLA YCN724 and UFLA YCN727 in the middle (312 h) and at the end (720 h) of fermentation. *Saccharomyces cerevisiae* UFLA YCN727 and UFLA YCN724 were inoculated as culture starters, but this species was also detected at all fermentation times in the other treatments. Beyond the species used as inoculum, other yeast species were detected during coffee fermentation *Debaromyces hansenii*, *Cystofilobasidium ferigula* and *Trichosporon cavernicola* (Fig. 2 and Table 1).

The bacterial diversity as revealed by PCR-DGGE bands consisted of 10 distinct bacterial species belonging to eight genera (Fig. 3 and Table 1). *Weissella soli*, *Leuconostoc mesenteroides*, *Gluconobacter oxydans*, *Pseudomonas aeruginosa*, *Pantoea eucrina*, *Erwinia toletana*, *E. tasmaniensis*, *Klebsiella oxytoca* and *Morganella morganii* were identified. The PCR-DGGE profile indicated an increase in the diversity of the bacterial populationfrom312 h, for treatments inoculated with yeast.

3.3. Chemical analyses

The organic acids: citric, malic, succinic, lactic and acetic were detected and quantified by HPLC (Table 2). Lactic, acetic and succinic acids were predominant and detected throughout the fermentation. Citric and malic acid were detected in the initial fermentation (Table 2). Oxalic, tartaric, butyric and propionic acid were not detected in any of the samples analyzed.

Table 2 Organic acids present in coffee semi-dry processing inoculated with yeast and the control at the beginning (0 h), intermediate (312 h) and end (720 h) of fermentation.

		Organic acids (g/Kg)				
Treatments	**Hours**	**Citric**	**Malic**	**Succinic**	**Lactic**	**Acetic**
[a]Control	0	1.43	2.08	1.12	nd	nd
Control	312	[b]nd	0.01	0.43	0.11	nd
Control	720	nd	nd	0.94	0.05	0.01
S. cerevisiae UFLA YCN727	0	0.57	0.15	0.29	0.73	nd
S. cerevisiae UFLA YCN727	312	0.02	nd	0.94	0.34	0.34
S. cerevisiae UFLA YCN727	720	nd	nd	0.47	0.22	0.10
S. cerevisiae UFLA YCN724	0	0.46	0.23	0.93	nd	nd
S. cerevisiae UFLA YCN724	312	0.06	nd	0.52	0.17	0.08
S. cerevisiae UFLA YCN724	720	nd	nd	0.10	0.07	0.21
C. parapsilosis UFLA YCN448	0	0.87	0.20	0.94	0.63	nd
C. parapsilosis UFLA YCN448	312	0.04	0.03	1.65	0.36	0.03
C. parapsilosis UFLA YCN448	720	nd	nd	0.89	0.14	0.04
P. guilliermondii UFLA YCN731	0	0.83	0.22	0.77	0.02	0.88
P. guilliermondii UFLA YCN731	312	0.13	nd	0.07	0.30	0.13
P. guilliermondii UFLA YCN731	720	nd	nd	0.55	0.12	0.06

The samples showed a standard deviation around 0.033.
[a]Control= without inoculum.[b]nd = not detected.

Forty-seven volatile compounds were identified and quantified with HS-SPME/GC in green and roasted coffee, including 3 ketones, 15 alcohols, 5 aldehydes, 7 acids, 10 esters, 5 furans, 1 phenol and 1 diether (Table 3).

Table 3 Relative percentage of volatile compounds identified in coffee inoculated with yeast and the control in green and roasted coffee.

Compounds	**Green coffee (%)**					**Roasted coffee (%)**				
	Control[a]	**UFLA YCN724[b]**	**UFLA YCN731[c]**	**UFLA YCN448[d]**	**UFLA YCN727[e]**	**Control**	**UFLA YCN724**	**UFLA YCN731**	**UFLA YCN448**	**UFLA YCN727**
Diether										
1.1-dietoxyethane	0.61	0.75	0.81	0.55	0.76	0.30	0.32	0.30	0.25	0.22
Ketones										
2,3-butanedione	nd[f]	0.60	0.81	nd	1.07	0.02	0.03	0.04	0.03	0.03
2-nonanone	nd	nd	nd	nd	nd	1.65	1.88	1.94	1.81	1.88
Verbenone	0.91	0.21	nd	0.46	0.62	1.06	1.18	1.30	1.22	1.27
Total ketones	0.91	0.81	0.81	0.46	1.69	2.73	3.09	3.28	3.06	3.18
Alcohols										
Methanol	0.86	0.28	0.22	0.19	nd	0.75	0.87	0.81	0.64	0.57
1-propanol	1.54	2.61	0.95	1.94	1.56	0.04	0.04	0.04	0.03	0.05
1-butanol	2.22	0.54	0.27	0.53	4.22	0.07	0.03	0.01	nd	nd
3-methyl-1-butanol	nd	0.35	nd	nd	nd	0.02	nd	nd	0.01	nd
1-pentanol	nd	nd	nd	nd	nd	5.29	5.67	5.78	6.10	5.43
2-heptanol	nd	0.33	nd	0.11	0.08	2.67	2.79	2.90	3.08	2.85
3-methyl-1-pentanol	1.07	2.34	0.90	1.12	1.44	nd	nd	nd	nd	nd
1-hexanol	2.43	4.93	nd	nd	nd	nd	nd	nd	nd	nd

Table 3(continued)

Linalool	nd	nd	nd	nd	nd	0.09	0.07	0.08	0.08	0.08
1,2-propanediol	nd	nd	nd	nd	nd	0.29	0.42	0.28	0.23	0.25
a-Terpineol	nd	nd	nd	nd	nd	0.04	0.05	0.04	0.04	0.05
b-Citronellol	nd	nd	nd	nd	nd	0.25	0.22	0.28	0.28	0.28
Geraniol	nd	nd	nd	nd	nd	0.03	0.04	0.07	0.05	0.05
2-phenylethanol	nd	nd	nd	nd	nd	0.05	0.07	0.08	0.05	0.06
Menthol	1.62	0.96	0.75	0.79	0.16	nd	0.04	0.16	0.14	0.16
Total alcohols	9.74	12.34	3.09	4.68	7.46	9.59	10.31	10.53	10.73	9.83
Aldehydes										
Acetaldehyde	0.61	0.69	0.52	0.45	0.52	0.51	0.61	0.49	0.33	0.38
Hexanal	19.81	17.20	12.81	12.58	21.39	nd	nd	0,01	nd	nd
Octanal	nd	nd	nd	nd	nd	0.30	0.31	0.26	0.32	0.36
Nonanal	0.73	0.39	nd	nd	nd	nd	nd	nd	nd	nd
Decyl aldehyde	nd	nd	nd	nd	nd	0.24	0.31	0.32	0.27	0.30
Total aldehydes	21.15	18.28	13.33	13.03	21.91	1.05	1.23	1.08	0.92	1.04
Acids										
Isobutyric acid	nd	nd	nd	nd	nd	11.62	11.53	10.50	9.54	10.34
Butyric acid	nd	nd	nd	nd	nd	0.10	0.13	0.14	0.11	0.12
Hexanoic acid	nd	nd	nd	nd	nd	0.06	0.06	0.07	0.08	0.05
Heptanoic acid	nd	nd	nd	nd	nd	nd	0.03	0.05	0.03	0.01
Octanoic acid	nd	nd	nd	nd	nd	nd	nd	nd	0.02	nd
Nonanoic acid	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.02
Decanoic acid	nd	nd	nd	nd	nd	0.30	nd	0.21	0.35	0.20
Total acids	0	0	0	0	0	12.08	11.75	10.97	10.13	10.74

Table 3(continued)

Esters										
Ethyl acetate	0.29	0.28	0.25	0.23	0.26	0.01	0.03	0.01	0.02	nd
Propyl acetate	6.67	6.21	3.86	3.88	4.78	0.55	0.66	0.59	0.56	0.57
Isobutyl acetate	nd	0.57	0.47	0.71	0.43	nd	nd	0.03	nd	nd
Ethyl butyrate	0.52	0.31	0.40	1.46	1.01	0.10	0.07	0.12	0.08	0.07
Isoamyl acetate	0.45	nd	nd	1.55	0.90	nd	nd	nd	nd	nd
Ethyl octanoate	0.09	nd	nd	nd	nd	0.08	0.01	nd	nd	nd
Diethyl malonate	nd	nd	nd	nd	nd	0.74	0.18	0.43	0.73	0.50
Phenyl acetate	nd	nd	nd	nd	nd	0.47	0.75	0.52	0.45	0.54
Propyl butyrate	0.23	nd	nd	0.34	nd	0.08	0.11	0.08	0.01	0.08
Diethylsuccinate	nd	nd	nd	nd	nd	0.09	0.12	0.10	0.08	0.20
Total esters	8.25	7.37	4.98	8.17	7.38	2.12	1.93	1.88	1.93	1.96
Phenol										
Guaiacol	nd	nd	nd	nd	nd	0.79	0.39	0.26	0.75	0.79
Furans										
Furfuryl alcohol	nd	nd	nd	nd	nd	14.87	16.25	14.43	15.64	15.72
Furfuryl acetate	nd	nd	nd	nd	nd	3.76	4.97	3.73	3.19	3.70
Furfuryl propionate	nd	nd	nd	nd	nd	0.37	0.48	0.43	0.35	0.38
Furfural	0.09	nd	nd	nd	nd	15.43	11.85	12.64	13.40	12.72
5-methylfurfural	1.86	3.08	2.24	2.37	2.84	0.05	0.03	0.03	0.04	0.04

Table 3(continued)

Total furans	1.95	3.08	2.24	2.37	2.84	34.48	33.58	31.26	32.62	32.56
Total GC area	26513	22432	20774	31792	23727	384449	445944	468765	498158	478573

[a]Control= without inoculum

[b] UFLA YCN724= *S.cerevisiae* UFLA YCN724

[c] UFLA YCN731= *Pichia guilliermondii* UFLA YCN731

[d]448= *Candida parapsilosis* UFLA YCN448

[e]727= *Saccharomyces cerevisiae* UFLA YCN727

[f] nd= not detected

Twenty-three compounds were found in green coffee. Among these, hexanal was present in the largest amount, followed by propyl acetate. Green coffee was richest in aldehyde, ester and alcohol (Table 3). Forty-two volatile compounds were found in roast coffee. The furans were the largest group identified, followed by acids and alcohols (Table 3). Furfuryl alcohol, furfural, isobutyric acid and 1-pentanol were the main compounds detected in roasted coffee (Table 3). The sample inoculated with UFLA YCN731 showed a smaller total area of peaks in the green coffee, which corresponds to a lower concentration of volatile compounds, while for roasted coffee, the lowest concentration was found in the control. The treatment inoculated with UFLA YCN448 had the greatest concentration of volatiles in both grains (Table 3).

Eighteen compounds were identified in both green and roasted coffee, but not in the same quantities. Some compounds, such as furfural, verbenone, 2-heptanol, and methanol, increased in quantity after roasting. Compounds that do not have a thermal origin, such as esters, decreased after roasting. These included ethyl acetate, propyl acetate, isobutyl acetate, and ethyl butyrate.

3.4. Sensory analyses

The results of the taste-test analysis are shown in Table 4. The coffee control and the coffee inoculated with UFLA YCN448 and UFLA YCN727 scored 80 points overall, which indicates very good coffee quality. The coffee inoculated with UFLA YCN724 and UFLA YCN731 had a total score of 79.33 and 74.17, respectively, indicating that they were also good coffee.

Table 4 Notes on the sensory analysis attributes for each treatment.

Attributes	Control[a]	*S.cerevisiae* UFLA YCN724	*P. guilliermondii* UFLA YCN731	*C. parapsilosis* UFLA YCN448	*S. cerevisiae* UFLA YCN727
Fragrance	7.27	7.17	7.25	7.33	7.42
Flavor	7.42	7.17	7.00	7.33	7.33
Aftertaste	7.25	7.00	6.83	7.17	7.17
Acidity	7.50	7.08	7.17	7.25	7.00
Body	7.42	7.17	6.92	7.25	7.17
Uniformity	10.00	9.33	6.00	9.33	10.00
Balance	7.17	7.08	6.83	7.00	7.33
Cleanliness	9.33	10.00	9.33	10.00	10.00
Sweetness	10.00	10.00	10.00	10.00	10.00
Score	7.58	7.33	6.83	7.33	7.67
Total Score	80.93	79.33	74.17	80.00	81.08

[a]Control= without inoculum.

The TDS curves of the five coffee treatments are presented in Fig. 4. The visual inspection of TDS curves showed a significant difference between the control and the treatment with inoculum, which was first

perceived as caramel. The coffee inoculated with UFLA YCN724 was characterized by perception of caramel for a very short time, followed by bitterness. Unlike any other treatment, it was dominated by an herbaceous sensation at the end. The coffee inoculated with UFLA YCN731 had a caramel flavor and was slightly acidic at the end. The coffee inoculated with UFLA YCN448 had a caramel flavor after 5s, which dominated until bitterness was perceived. The coffee inoculated with UFLA YCN727 had the best results. At the beginning, it was dominated by caramel, followed by bitter, and then finished with caramel. The control coffee had an acidic sensation in the beginning; after 5s, it was dominated by bitterness that was perceived by the end of the analysis with high intensity.

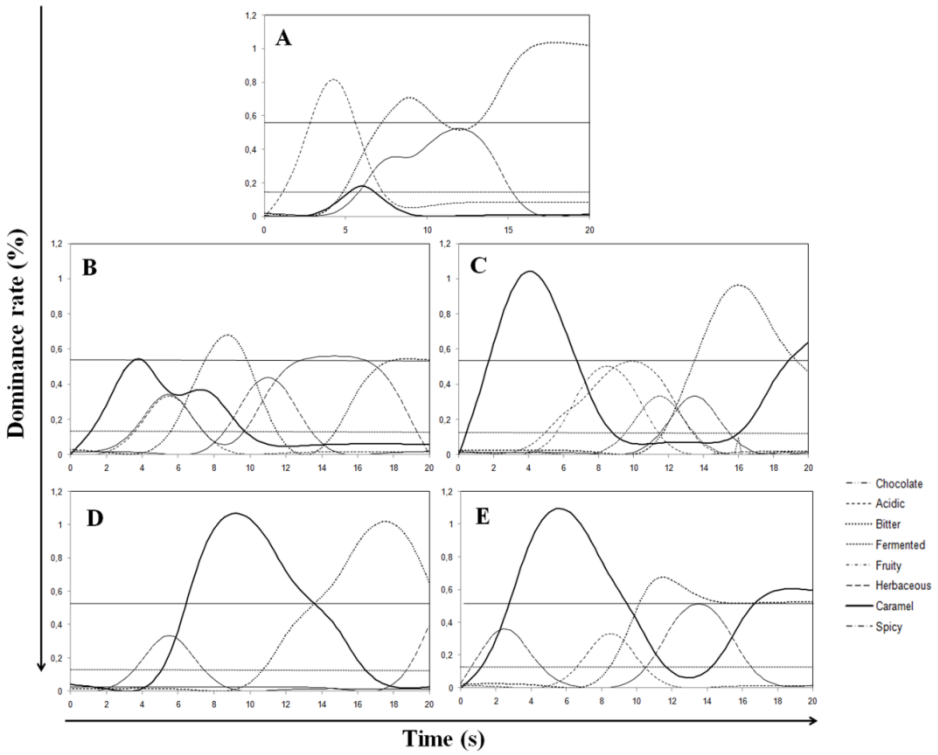

Fig. 4 TDS curves of coffee (A) Control, (B) *Saccharomy cescerevisiae* UFLA YCN724, (C) *Pichia guilliermondii* UFLA YCN731, (D) *Candida parapsilosis* UFLA YCN448, and (E) *S. cerevisiae* UFLA YCN727. Significance level (⁻) and chance level (···).

3.5 Multivariate analysis

The principal component analysis allowed the correlation between volatile compounds and the four treatments inoculated with yeast and the control in green beans (Fig. 5A) and after roasting (Fig. 5B). The first two principal components account for 67.87% of the total variability in the data set (Fig. 5A). PC1, which explained 42.25% of the total variability, characterized the separation of the treatment into two groups: one formed by the coffee inoculated with *S. cerevisiae* UFLA YCN727 and *P. guilliermondii* UFLA YCN731 in the positive part of the PC1, and *S. cerevisiae* UFLA YCN724, *C. parapsilosis* UFLA YCN448 and the control in the negative part of the PC1.

The treatment inoculated with UFLA YCN727 was mainly characterized by the compounds 2,3-butanedione, isobutyl acetate and 3-methyl-1-butanol, and the treatment inoculated with UFLA YCN731 by 1.1-dietoxyethane. The treatments inoculated with UFLA YCN448 and UFLA YCN724 contained similar compounds and were located in the same region of the graph. These two treatments were mainly characterized by 5-methylfurfural, ethyl butyrate, 1-propanol, 2-heptanol,

and 3-methyl-1-pentanol. The control was the most different sample and was mainly characterized by methanol and furfural (Fig. 5A).

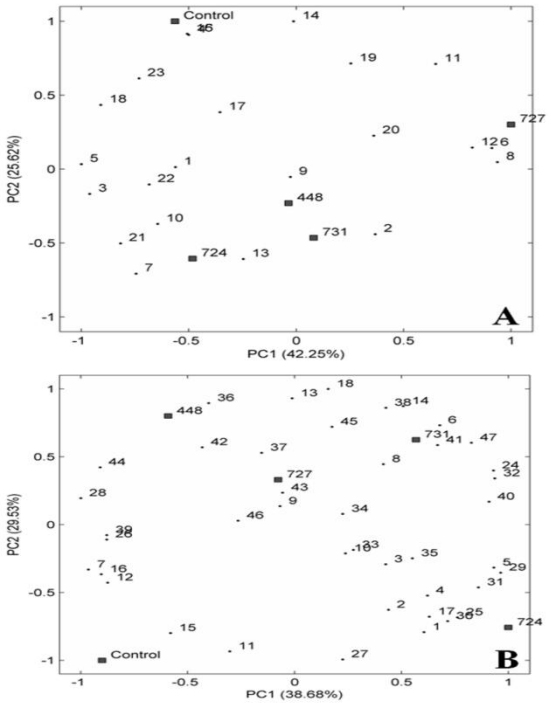

Fig. 5 Principal component analysis (PCA) of volatile compounds identified in (A) green and (B) roasted coffee. Spontaneous fermentation (control), and coffee inoculated with *Saccharomyces cerevisiae* UFLA YCN727, *S.cerevisiae* UFLA YCN724, *Candida parapsilosis* UFLA YCN448, or *Pichia guilliermondii* UFLA YCN731. (1) Acetaldehyde, (2) 1.1-diethoxyethane, (3) Ethyl acetate, (4) Methanol, (5) Propyl acetate, (6) 2,3-butanedione, (7) 5-methylfurfural, (8) Isobutyl acetate, (9) Ethyl butyrate, (10) 1-propanol, (11) 1-butanol, (12) 3-methyl-1-butanol, (13) 2-heptanol, (14) Verbenone, (15) Ethyl octanoate, (16) Furfural, (17) Propyl butyrate, (18) Menthol, (19) Hexanal, (20) Isoamyl acetate, (21) 3-methyl-1-pentanol, (22) 1-hexanol, (23) Nonanal, (24) Decyl aldehyde, (25) Furfuryl acetate, (26) Linalool, (27) Isobutyric acid, (28) Diethyl malonate, (29) Furfuryl propionate, (30) 1,2-propanediol, (31) Phenyl acetate, (32) Butyric acid, (33) Furfuryl alcohol, (34) Diethylsuccinate, (35) a-Terpineol, (36) b-Citronellol, (37) Hexanoic acid, (38) Geraniol, (39) Guaiacol, (40) 2-phenylethanol, (41) Heptanoic acid, (42) Octanoic acid, (43) Nonanoic acid, (44) Decanoic acid, (45) 1-pentanol, (46) Octanal, (47) 2-nonanone.

In the roasted coffee, the first two principal components accounted for 68.21% of the total variability (Fig. 5B). The compounds present in roasted coffee inoculated with *P. guilliermondii* UFLA YCN731 and *S. cerevisiae* UFLA YCN724 were localized in the positive part of the PC1, which explained 38.68% of the total variability. The roasted coffee inoculated with UFLA YCN731 was mainly characterized by 2,3-butanedione, isobutyl acetate, heptanoic acid and 2-nonanone. The roasted coffee inoculated with UFLA YCN724 contained mainly acetaldehyde, propyl butyrate, furfuryl acetate, 1,2-propanediol and phenyl acetate (Fig. 5B).

In the region negative of PC1 and positive of PC2 , the coffee samples inoculated with *C. parapsilosis* UFLA YCN448 and *S. cerevisiae* UFLA YCN727 were characterized by ethyl butyrate, b-citronellol, hexanoic acid, octanoic acid, nonanoic acid and octanal, and the control was mainly characterized by 1-butanol and ethyl octanoate (Fig. 5B).

4. Discussion

Analysis of the DGGE profiles showed that starter cultures *S. cerevisiae* UFLA YCN727, *S. cerevisiae* UFLA YCN724, *C. parapsilosis* UFLA YCN448 and *P. guilliermondii* UFLA YCN731 had multiple band patterns (Fig. 2). According to Nübel et al. (1999), more than one band is due to sequence heterogeneity between multiple copies of 16S rDNA. In the control, *C. parapsilosis* and *P. guilliermondii* were detected in the middle (312 h) and at the end (720 h) of fermentation (Fig. 2), despite not being inoculated. These yeasts have been described as naturally present during coffee fermentation (Silva et al., 2008). However, these species were not detected in the beginning of fermentation. The presence of these species in the middle and late stages of fermentation could be probably due to changes in the environment such as pH value and moisture (Vilela et al., 2010).

Other microbial species identified in this study by DGGE (Fig. 2 and Table 1), beyond the starter cultures were reported in different coffee processing (Avallone et al., 2001; Masoud et al., 2004; Silva et al., 2008, Vilela et al., 2010). Among these species the yeast *Debaryomyces hansenii* was the predominant species in coffee processed by via natural

or dry (Silva et al., 2008). The presence of some yeast genera, such as *Pichia*, not only contributes to the degradation of pectin in coffee (Masoud and Jespersen, 2006), but also can inhibit the growth of fungi that produce ochratoxin A (OTA), such as *Aspergillus ochraceus* (Masoud and Kaltoft, 2006; Massawe and Lifa, 2010; Ramos et al., 2010).

The species *Mitchella repens* (bands 14 and 20) that was present at the end of fermentation in all treatments is a plant that belongs to the family Rubiaceae, to which the genus *Coffea* belongs. This species was reported by Masoud et al. (2004) in coffee fermentation processed via the wet method and by Evangelista et al. (2013) in natural (dry) coffee fermentation.

The bacterial profile in the PCR-DGGE analysis and the organic acids were similar in the treatments inoculated with UFLA YCN724, UFLA YCN727 and UFLA YCN731 (Table 2 and Fig. 3). The presence of organic acids during fermentation may be due to coffee composition, such as citric acid, or may be associated with the metabolism of the microorganisms (Silva et al., 2013), as *Leuconostoc mesenteroides* (b and 2 and 8). This heterofermentative lactic acid bacterium (LAB) produces

lactic and acetic acids (Avallone et al., 2001). Lactic and acetic acids (Table 2) were found in highest concentration in the treatments inoculated with UFLA YCN727 and UFLA YCN448 in which *Leuconostoc mesenteroides* was present (Fig. 3).

The lactic, succinic, acetic and malic acids detected in this work (Table 2), in general increase acidity in the final product (Buffo and Cardelli-Freire, 2004), and its presence is desirable, butbutyric and propionic acids may impair the flavor of beverage are not desirable. The starter cultures *S. cerevisiae* UFLA YCN727, *S. cerevisiae* UFLA YCN724, *C. parapsilosis* UFLA YCN448 and *P. guilliermondii* UFLA YCN731, did not favor the production of undesirable acids such as butyric and propionic acid, therefore they influenced positively the final quality of the coffee (Table 2), being very promising for use as inoculants in coffee fermentation.

Data from HS-SPME/GC showed that the starter cultures lead to an increase of volatile compounds in roasted coffee (Table 3 and Fig. 5B). Yeasts used in this study as starter culture, were reported in a previous work as producers ofpectinolytic enzymes and therefore aid in the degradation of the pectin present in coffee pulp and mucilage by

producing metabolites that diffuse into the interior of the coffee beans, favoring the formation of the flavor of the final beverage (Silva et al., 2013). Differences between the volatile compounds found in green and roasted coffee were observed (Table 3). Alcohols, acids and furans were present in high concentrations after roasting. It is known that the volatile fraction of coffee beans develops primarily in the form of alcohols, acids, esters and aldehydes (Gonzalez-Rios et al., 2007). Compounds of these classes are associated with flavor during the fermentation of coffee, such as 1-pentanol, 2-phenylethanol, acetaldehyde, hexanal, ethyl acetate, guaiacol, furfuryl alcohol, furfural and others that were detected in this work (Table 3).

Furfural was detected in roasted coffee samples (Table 3) and the bitter flavor was perceived after roasting in all treatments (Fig. 4 and Fig. 5), suggesting that this compound might be responsible or influenced the bitter flavor found in the final beverage (Gonzalez-Rios et al., 2007). The temporal dominance of sensation analysis showed that only the control had an acidic flavor (Fig. 4). The coffee inoculated with UFLA YCN448, UFLA YCN724, UFLA YCN727 and UFLA YCN731 had a caramel flavor at the beginning of the analysis, followed by the bitter flavor

(Fig.4). The caramel flavor detected in the treatments with starter cultures is a desirable sensation (Gonzalez-Rios et al., 2007). The use of these yeast cultures demonstrated favorable for the production of coffee with different flavors, when compared to control that did not show a distinctive flavor (Fig. 4).

Coffee inoculated with UFLA YCN448 and UFLA YCN727 had the highest score in the taste-test. This result was confirmed by TDS analysis, which showed a dominance of caramel flavor in the beginning and a bitter aroma at the end of the analysis. The treatment inoculated with UFLA YCN731 and UFLA YCN724 did not reach a score of 80, but the TDS results were a dominant feeling of a caramel aroma with bitter and fermented herbal tastes (Fig. 4 and Table 4). These results suggest that the coffee inoculated with *S. cerevisiae* (UFLA YCN724) and *P. guilliermondii* (UFLA YCN731) can be used as blends, giving a special coffee with distinctive flavor. The TDS was an appropriate analysis to describe the sensory characteristics of the coffee, and the results were in agreement with the results of the taste-test.

In a previous study, Evangelista et al., (2013) observed that the use of starter cultures, the same used in this work, in coffee cherries

processed by natural or dry fermentation produced coffee beverages with a distinctive flavor. However, their results were different from those presented here. Although both studies used the same yeasts as starter cultures, they showed different flavors when the coffee cherries were processed by the semi-dry fermentation method. In natural fermentation, UFLA YCN448 inoculation produced coffee with caramel and herbaceous flavors, whereas coffee inoculated with UFLA YCN727 had a fruity flavor. When the same yeast strains were inoculated in depulped coffee cherries using the semi-dry fermentation method, the coffees inoculated with UFLA YCN448 and UFLA YCN727 had caramel and bitter flavors, as discussed above.

In conclusion, the results demonstrated that the use of yeasts as starter cultures is a good alternative for obtaining high quality coffee with a distinctive flavor. A coffee with a special aroma of caramel was maintained during fermentation, and the usual characteristics evaluated in coffee beverages were not impaired. The use of starter cultures in coffee fermentation is an interesting alternative for obtaining a differentiated product. *C. parapsilosis* UFLA YCN448 and *S. cerevisiae* UFLA YCN727 gave a better result in relation to sensory analysis in coffee

processed by the semi-dry method. However, futures studies should be performed to confirm the best strain to use and to evaluate the ability of yeast inocula to inhibit the growth of filamentous fungi.

Acknowledgements

The authors thank the Brazilian agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico of Brasil (CNPQ), Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for scholarships. We also thank the farm Fazenda Juliana located in Monte Carmelo city and other farms situated in Lavras in the state of Minas Gerais, Brazil for collecting samples.

References

Avallone, S., Guyot, B., Brillouet, J-Marc., Olguin, E., Guiraud, J-Pierre., 2001. Microbiological and Biochemical Study of Coffee Fermentation. Current Microbiology 42, 252-256.

Buffo, R. A., Cardelli-freire, C., 2004. Coffee flavour: an overview. Flavour and Fragrance Journal19 (2), 99-104.

Charles-Bernard, M., Kraehenbuehl, K., Rytz, A., Roberts, D. D., 2005. Interactions between volatile and onvolatile coffee components. 1. Screening of nonvolatile components. Journal Agriculture Food Chemistry 53, 4417-4425.

Cocolin, L., Bisson, L.F., Mills, D.A., 2000. Direct profiling of the yeast dynamics in wine fermentations. FEMS Microbiology Letters 189, 81-87.

Coltro, L., Mourad, A. L., Oliveira, P. A. P.L.V., Baddini, J. P. O. A., Kletecke, R. M., 2006. Environmental profile of Brazilian green coffee. International Journal of Life Cycle Assessment 11 (1), 16-21.

Duarte, W. F., Dias, D. R., Oliveira, J. M., Teixeira, J. A., Silva, J. B. A., Schwan, R. F., 2010. Characterization of different fruit wines made from cacao, cupuassu, gabiroba, jaboticaba and umbu. LWT - Food Science and Technology 43(10), 1564-1572.

Evangelista, S. R., Silva, C. F., Miguel, M. G. P. C., Cordeiro, C. S., Pinheiro, A. C. M., Duarte, W. F., Schwan, R. F., 2013. Improvement of coffee beverage quality by using selected yeasts strains during the fermentation in dry process. Food Research International (in press).

Gonzalez-Rios, O., Suarez-Quiroza, M. L., Boulanger, R., Barel, M., Guyot, B., Guiraud, J. P., Schorr-Galindo, S., 2007. Impact of ‘‘ecological’’ post-harvest processing on coffee aroma: II. Roasted coffee. Journal of Food Composition and Analysis 20, 297–307.

Masoud, W., Cesar, L. B., Jespersen, L., Jakobsen, M., 2004. Yeast involved in fermentation of *Coffea arabica* in East Africa determined by genotyping and by direct denaturating gradient gel electrophoresis. Yeast 21, 549–556.

Masoud, W., Jespersen, L., 2006. Pectin degrading enzymes in yeasts involved in fermentation of *Coffea arabica* in East Africa. International Journal of Food Microbiology 110, 291–296.

Masoud, W., Kaltoft, C. H., 2006. The effects of yeasts involved in the fermentation of *Coffea arabica* in East Africa on growth and ochratoxin A (OTA) production by *Aspergillus ochraceus*. International Journal of Food Microbiology 106, 229 – 234.

Massawe, G. A., Lifa, S. J., 2010. Yeasts and lactic acid bacteria coffee fermentation starter cultures. International Journal of Postharvest Technology and Innovation 2(1), 41-80.

Nübel, U., Garcia-Pichel, F., Kühl, M., Muyzer, G., 1999. Quantifying microbial diversity: Morphotypes, 16S rDNA genes and carotenoids of oxygenic phototrophs in microbial mats. Applied Environmental Microbiology 65, 422–430.

Nunes, C. A.; Pinheiro, A. C. M., 2012. Sensomaker Free user-friendly interface for sensory analysis. Available from: www.ufla.br/sensomaker (Accessed at: Out. 2012)

Ovreas, L., Forney, L., Daae, F. L., 1997. Distribution of Bacterioplankton in Meromictic Lake Saelenvannet, as Determined by Denaturing Gradient Gel Electrophoresis of PCR-Amplified Gene Fragments Coding for 16S rRNA. Applied and Environmental Microbiology 63(9), 3367–3373.

Petisca, C., Pérez-Palacios, T., Farah, A., Pinho, O., Ferreira, I. M. P. L. V. O., 2013. Furans and other volatile compounds in ground roasted and espresso coffee using headspace solid-phase microextraction: Effect of roasting speed. Food and Bioproducts Processing 91(3), 233-241.

Pineau, N., Schlich, P., Cordelle, S., Mathonnière, C., Issanchou, S., Imbert, A., Rogeaux, M., Etiévant, P., Koster, E., 2009. Temporal Dominance of Sensations: Construction of the TDS curves and comparison with time–intensity. Food Quality and Preference 20, 450-455.

Ramos, C. L.,Almeida, E. G., Pereira, G. V. D. M., Cardoso, P. G., Dias, E. S., Schwan, R. F., 2010. Determination of dynamic characteristics of microbiota in a fermented beverage produced by Brazilian Amerindians using culture-dependent and culture-independent methods. International Journal of Food Microbiology 140(2-3), 225-31.

Ramos, D. M. B., Silva, C. F., Batista L. R., Schwan, R. F., 2010. Inibição *in vitro* de fungos toxigênicos por *Pichia* sp. e *Debaryomyces* sp. isoladas de frutos de café (*Coffea arabica*). Acta Scientiarum. Agronomy 32 (3), 397-402.

Schwan, R. F., Silva C. F., Batista L. R., 2012. Coffee fermentation. In: Hui, Y. H . (Ed), Handbook of plant-based fermented food and beverage technology(pp 677–690). CRC Press, Boca Raton.

Schwan, R. F., Wheals, A. E., 2003. Mixed microbial fermentation of chocolate and coffee. In: Boekhout, T. R. V. (Ed), Yeasts in food (pp 426–459). Behr's Verlag, Hamburg.

Silva, C. F., Batista, L. R., Abreu, L. M., Dias, E. S., Schwan, R. F., 2008. Succession of bacterial and fungal communities during natural coffee (C*offea arabica*) fermentation. Food Microbiology 25(8), 951-7.

Silva, C. F., Schwan, R. F., Dias, E. S., Wheals, A. E., 2000. Microbial diversity during maturation and natural processing of coffee cherries of Coffea arabica in Brazil. International Journal of Food Microbiology 60(2-3), 251-60.

Silva, C. F., Vilela, D. M., Souza, C. C., Duarte, W. F., Dias, D. R., Schwan, R. F., 2013. Evaluation of a potential starter culture for enhance quality of coffee fermentation. World Journal of Microbiology & Biotechnology 29(2), 235-47.

SPECIALTY COFFEE ASSOCIATION OF AMERICA (SCAA) (2013). Available in: <http://www.scaa.org> Accessed at: April. 2013.

Vilela, D. M., Pereira, G. V. D. M., Silva, C. F., Batista, L. R., Schwan, R. F., 2010. Molecular ecology and polyphasic characterization of the microbiota associated with semi-dry processed coffee (*Coffea arabica L.*). Food Microbiology 27(8), 1128-35.

Wakeling, I. N., Macfie, H. J. H., 1995. Designing consumer trials balanced for first and higher orders of carry-over effect when only a subset of k samples from the may be tested. Food Quality and Preference 6, 299-308.

ARTIGO 3 Coffee wet processing in Brazil: microbiological, physico-chemical and sensorial characterization

Suzana Reis Evangelista[a,b], Maria Gabriela da Cruz Pedroso Miguel[b], Cristina Ferreira Silva[b], Rosane Freitas Schwan[b,]*

[a]Food Sciences Department, Federal University of Lavras, CEP 37200-000, Lavras, MG, Brazil

[b]Biology Department, Federal University of Lavras, CEP 37200-000, Lavras, MG, Brazil

*Corresponding author

x (+55) 35 3829 1100; e-mail: rschwan@dbi.ufla.br]

Running title: **Microbialcharacterization ofcoffeewet processing**

Artigo científico a ser submetido para o periódico: *International Journal of Food Microbiology*.

ABSTRACT

The microorganisms involved in coffee fermentation are bacteria, yeast, and filamentous fungi, which can all be influenced by several factors including the way in which the coffee cherries are processed: wet, semi-dry, or dry. This work aimed to evaluate the microbiota present during coffee wet processing in two distinct regions of Minas Gerais, Brazil: one farm in the South of Minas Gerais (Lavras = L) and another farm in the savannah region (Monte Carmelo = MC). The culture-dependent and -independent methods (PCR-DGGE) were used to evaluate the microbiota. Organic acids and volatile compounds were quantified via HPLC and HS-SPME/GC, respectively. Sensory analyses were evaluated using the temporal dominance of sensations (TDS).The population of bacteria was higher than that of yeast, and it was more diverse during the entire fermentation process in the two regions studied. *P.caribbica (*53%) and *H. uvarum* (35.57%) were the dominant yeasts in coffee wet processing at L farm, and *Torulaspora delbrueckii* (50%) was the dominant yeast at MC farm. The species *Staphylococcus warneri* (35.29%) and *Erwinia persicina* (19.61%) were the predominant bacteria at L farm, and *Enterobacter asburiae* (41.80%) and *Leuconostoc*

mesenteroides (24.59%) were the dominant species at MC farm. The main sugar detected at the beginning of the fermentation was fructose (3.18 g/Kg at L farm and 1.48 g/Kg at MC farm). Lactic acid was the principal acid detected; it showed increasing concentrations throughout the fermentation process, reaching 2.33 g/Kg at L farm and 1.40 g/Kg at MC farm by the end of the process. The volatiles' composition was similar for roasted coffee from the two different regions and furans, acids, and alcohol were the mains groups detected. An analysis of the TDS curves showed that coffee from L farm was dominated by citrus and herbaceus sensations and that the coffee from MC farm was dominated by citrus, herbaceous, and nut sensations. Evaluating the microbiota in the two regions (L farm and MC farm) was important in obtaining greater knowledge of the microbial species present during the wet processing of coffee. This work is the first to evaluate the microbiota present in wet coffee processing in Brazil, and the results can be used in futures studies to select starter cultures for wet processing.

Keywords: Arabica coffee, Wet processing, Microbiota, PCR-DGGE, Yeasts, Bacteria.

1. Introduction

Brazil is the largest producer and exporter of coffee, and due to the diversity of the production process, three types of coffee processing can be used (dry, semi-dry, and wet) to obtain different final coffees (MAPA, 2014).The State of Minas Gerais has four producing regions, which account for about 53% of all coffee produced in Brazil. Some coffee producers in the southern and central areas of Minas Gerais use wet processing (Sindcafé-MG, 2014). In wet processing, the coffee peel is removed mechanically and then taken to a tank with water to remove the mucilage. This processing provides a coffee with a different composition and sensory characteristics than coffee processed byte semi-dry or natural methods, which are alternatives used by the producer to suit different markets (Oliveira et al., 2012).

Microorganisms are naturally present during coffee processing and use the various compounds presents in the pulp and mucilage as nutrients to grow. They secrete organic acids and other metabolites that may affect the final sensory characteristics of the beverage (Schwan et al., 2012). This microbiota may vary according to several factors: the regional characteristics (temperature and altitude), the grain characteristics

(humidity, pH, and composition), and the type of processing used**.** It is important to know the microbiota present during the processing of coffee**,** especially when selecting starter cultures that can be used in producing differentiated final products and inhibiting the growth of mycotoxigenic fungi (Evangelista et al., 2013; Masoud and Jespersen, 2006; Masoud et al., 2005; Massawe and Lifa, 2010; Silva et al., 2012).

Studies have been performed to evaluate the microbiota present in coffee wet processing in various countries. An early study was conducted by Agate and Bhat (1966). It evaluated the predominant microbiota in wet coffee in India and described the presence of the following yeast species: *Saccharomyces marxianus*, *S.bayanus, S. cerevisiae* var. *ellipsoideus*, and *Schizosaccharomyces* sp. Frank et al. (1965) determined the bacteria population of wet coffee fermented in the Kona region of the island of Hawaii, detecting mainly the *Erwinia*, *Paracolobactrum*, and *Escherichia* genera.

The aerobic mesophilic, lactic acid bacteria, and yeast groups were the microbial groups detected during wet processing in Mexico. *Erwinia*, *Klebsiella* and *Leuconostoc* were the bacteria identified, and

Kloeckera, *Candida*, and *Cryptococcus* were the yeasts identified (Avallone, 2001).

The PCR-DGGE method has been used to assess the yeast population during the fermentation of coffee in wet processing in Tanzania (Masoud et al., 2004). The predominant yeasts found were *Pichia kluyveri*, *Pichia anomala*, *Hanseniaspora uvarum*, *Kluyveromyces marxianus*, *Candida pseudointermedia*, *Pichia ohmeri*, *Torulaspora delbrueckii*, *Saccharomyces cerevisiae* and *Candida xestobii.* Durand et al. (2013) evaluated the dynamics of coffee microbial populations (fungi and yeast) during the different stages of postharvest treatment (the wet and dry processes) in Mexico at various altitudes.This was an efficient technique.

Coffee is produced in countries that have varying conditions, such as climate and altitude, which may influence the microbiota involved during fermentation (Leong et al., 2014). Studies are needed to gain knowledge of the microbiota present in coffee processing for each producing region. This is the first study to evaluate the microbiota present during coffee wet processing in Brazil. Our aim was to study the microbial diversity involved during coffee wet processing , using culture-

dependent and culture-independent methods, as well as evaluate the metabolites present during the fermentation process, using headspace solid-phase microextraction/gas chromatography (HS-SPME/GC) and high-performance liquid chromatography (HPLC). An additional aim was to assess the sensory profile of the coffee.

2. Materials and Methods

2.1. Sampling

Coffee cherries of *Coffea arabica* L. var. Acaiá were harvested at the mature stage (red cherries) and processed using the wet method. The experiments were performed in two geographically different regions of Minas Gerais, at a farm located in Monte Carmelo (MC), 870 m above sea level in the savannah (Cerrado) region, and at a farm located in Lavras (L), 919 m above sea level in the Atlantic Forest region.

2.2. Processing

The cherries (60 kg) were mechanically depulped in a horizontal machine (model BDSV-04; Pinhalense, São Paulo, Brazil), followed by fermentation and mucilage removal in a tank with 60 liters of water. In

the experiment conducted at Lavras, the depulped coffee fruits were allowed to ferment in the tank for 40 hours, and the environmental temperature varied between 14 and 23 °C. At the Monte Carmelo farm, the grains remained in the tank for 28 hours, and the ambient temperature varied between 20 and 28 °C. After fermentation, the grains were placed in suspended platforms for drying until they reached 11% moisture. Samples were collected aseptically in sterile plastic bags during fermentation and drying and then transferred to the Microbiology Laboratory of the Federal University of Lavras (UFLA) for microbiological and physicochemical analyses.

2.3 Characterization and identification of microbiota

2.3.1 Quantification, isolation, and phenotypic characterization

Ten grams of coffee bean were added to 90 mL of sterile peptone water [in g/L: 1 bacteriological peptone (Himedia)], homogenized for 10 min in a Stomacher (Mayo Homogenius HG 400, São Paulo, Brazil), and used for serial dilution. The bacteria population was enumerated via inoculation on culture media PCA [in g/L: 5 tryptone (Himedia), 2.5 yeast extract (Merck), 1 glucose (Merck), 15 agar (Merck)], and the yeasts were

enumerated via inoculation on culture media YEPG [in g/L: 20 glucose (Merck), 10 yeast extract (Merck), 10 peptone soy (Himedia), 20 agar (Merck), pH 3.5].The plates were incubated at 28 °C for 48 hours. Subsequent to incubation, the number of colony-forming units (CFUs) was counted, and the morphological characteristics (cell size, cell shape, edge, color, and brightness) of the different colony types were characterized. The square root of the number of colonies counted for each morphotype were purified by streaking on new agar plates (same culture media used for plating) (Senguna et al., 2009). The pure cultures were stored at -80 °C in YEPG broth containing 20% glycerol (w/w).

The phenotypic characterization of the bacterial colonies was performed using conventional microbiological methods, including Gram staining, the determination of catalase and oxidase activities, motility tests, growth in culture media with 1% and 5% (w/v) NaCl (salt tolerance), protease production, spore formation, and the ability to ferment glucose, sucrose, and xylose, as recommended in *Bergey's Manual of Determinative Bacteriology* (Holt et al., 1994). Yeast colonies were physiologically characterized via the determination of their morphology; fermentation of sucrose, glucose, and fructose; Diazonium

Blue B test (DBB); and hydrolysis of urea, as described by Kurtzman et al. (2011).

2.3.2 Genotypic identification

The isolates were grouped by phenotypic characteristics, and a representative sample of each phenotypic group (236 isolates) was subjected to molecular analyses. The genotypic characterization of the selected isolates was first performed via rep-PCR, as in Nielsen et al. (2007), and subsequently via sequencing.

The bacterial and yeast cultures were grown under appropriate conditions, collected from agar plates with a sterile pipette tip, and resuspended in 50 μL of sterile Milli-Q water. The suspension was heated for 10 min at 95 °C, and 3 μL was used as a DNA template in PCR experiments.

The fingerprints of the genomic DNA were obtained via the PCR amplification of repetitive bacterial and yeast DNA elements (rep-PCR) using the $(GTG)_5$ primer, as described by Nielsen et al. (2007). Amplified PCR products were separated via 2% (w/v) agarose gel electrophoresis at

70 V for 3 h, and the images were visualized and photographed using a transilluminator LPixImage (LTB 20 X 20 HE, LPix®, Brazil).

A cluster analysis was performed using BioNumerics software (version 4.50).Similarities were calculated using a DICE correlation coefficient and UPGMA algorithm, as previously described (Parkouda et al., 2010).

The bacteria and yeasts representative of each cluster (50 isolates of bacteria and 63 yeasts) were subjected to 16S rRNA gene and internal transcribed spacer (ITS) region gene sequencing, respectively. The amplification of the 16S rRNA gene used the primers 27F (5’ AGA GTT TGA TYM TGG CTC AG 3’) and 1512R (5’ ACG GYT ACC TTG TTA CGA CTT 3’) (Devereux & Willis, 1995).The ITS region was amplified using primers ITS1 (5’-TCC GTA GGT GAA CCT GCG G- 3’) and ITS4 (5’-TCC TCC GCT TAT TGA TAT GC-3’) (Nielsen et al., 2007). Amplified PCR products were sent for sequencing at the Advanced Genetics Technologies Center – AGTC (Kentucky, USA); the ABI3730 XL automatic DNA sequencer was utilized.

The sequences were aligned using the BioEdit 7.7 sequence alignment editor and were compared to the GenBank database using the

Basic Local Alignment Tool (BLAST) program (National Center for Biotechnology Information, Bethesda, MD) for the identification of isolates at the genus and species level.

2.4. Microbial community analysis through PCR-DGGE

2.4.1. Total DNA extraction and PCR analysis

Three grams of sample were mixed with 5 mL of Milli-Q water for 10 min, followed by centrifugation at 100×g for 10 min at 4 °C. The pellet was used for DNA extraction. The total DNA was extracted from samples taken during fermentation and drying using the "DNA Purification from Tissues" protocol [QIAamp DNA Mini Kit (Qiagen, Hilden, Germany)] in accordance with the manufacturer's instructions. The DNA from the bacterial community was amplified with the primers 338fgc (5′ CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG 3′) (the GC clamp is underlined) and 518r (5′ ATT ACC GCG GCT GCT GG 3′), which span the V3 region of the 16S rRNA gene (Ovreas et al., 1997).A fragment of the D1-region of the 26S rRNA gene was amplified with the eukaryotic universal primers NL1GC (5′ CGC CCG CCG CGC GCG

GCG GGC GGG GCG GGG GCA TAT CAA TAA GCG GAG GAA AAG 3′) (the GC clamp is underlined) and LS2 (5′ ATT CCC AAA CAA CTC GAC TC 3′), which amplified a fragment of approximately 250 bp (Cocolin et al., 2000). PCR was carried out in a final reaction volume of 25 µL, containing 0.625 U Taq DNA polymerase (Promega, Milan, Italy), 2.5 µL 10× buffer, 0.1 mM dNTPs, 0.2 mM of each primer, 1.5 mM $MgCl_2$, and 1 µL of extracted DNA. The amplification was performed according to Ramos et al. (2010). Aliquots (3 µL) of the amplification products were analyzed via electrophoresis on 1% agarose gels before they were used for DGGE.

2.4.2. PCR-DGGE analysis

The PCR products were separated in polyacrylamide gels [8% (w/v) acrylamide: bisacrylamide (37.5:1)] in 1× TAE buffer with a DCode system apparatus (BioRad Universal Dcode Mutation Detection System, Richmond, CA, USA). Denaturation gradients were used that varied from 15 to 55% for the bacterial products (100% corresponded to 7 M of urea and 40% [v/v] formamide) and from 20 to 60% for the yeast products. Electrophoresis was conducted at a constant voltage of 130 V

for 6 hours (for bacteria and yeast) and at a constant temperature of 60 °C. Following electrophoresis, the gels were stained with SYBR-Green I (Molecular Probes, Eugene, UK) (1:10.000 v/v) for 30 min. The images were visualized and photographed using a transilluminator LPixImage (LTB 20 X 20 HE, LPix®, Brazil).

2.4.3. DGGE band sequencing

Selected bands from the PCR–DGGE gels were excised with a sterile blade and placed in 50 μL of sterile Milli-Q water at 4 °C overnight to allow the DNA to diffuse out of the polyacrylamide matrix. The samples were then amplified using the primers 338fgc and 518r for bacteria and NL1 and LS2 for yeast. The PCR products were purified and sequenced at the Advanced Genetics Technologies Center – AGTC (Kentucky, USA); the ABI3730 XL automatic DNA sequencer was utilized. The sequences were aligned using the BioEdit 7.7 sequence alignment editor and were compared to the GenBank database using the BLAST algorithm (National Center for Biotechnology Information, Maryland, USA).

2.5. Chromatographic analysis of the coffee

2.5.1. Characterization of organic acids and carbohydrates via HPLC

The organic acids (malic, lactic, acetic, butyric, propionic, citric, oxalic, succinic, and tartaric acids) and carbohydrates (glucose, sucrose, and fructose) were analyzed using a high-performance liquid chromatography system (Shimadzu Corp., Japan) equipped with a dual detection system consisting of a UV–Vis detector (SPD 10Ai) and a refractive index detector (RID-10Ai). Three grams of sample were mixed with 5 mL of Milli-Q water for 10 min, and the fluids were centrifuged twice at 100×g for 10 min at 4 °C. The samples were microfiltered through a 0.2 µm cellulose acetate filter and directly injected (20 µl) onto the chromatography column. A Shimpack SCR-101H (7.9 mm × 30 cm) column that was operated at 50 °C for acids and 30 °C for carbohydrates was used. Ultra-pure water (pH adjusted to 2.1 with perchloric acid) was used in the mobile phase, and the flow rate was 0.6 mL/min. The acids were detected via UV absorbance (210 nm), and the sugars were detected via RID. Identification was performed via a comparison with the retention times of authentic standards. The quantification of compounds was performed using calibration curves constructed with different

concentrations of standard compounds[glucose, fructose, malic, propionic, and citric acids were purchased from Merck (Germany); sucrose, lactic, oxalic, and tartaric acids were purchased from Sigma-Chemical (EUA); acetic and succinic acids were purchased from Sigma-Aldrich (Germany); butyric acid was purchased from Riedel-deHaen (Germany)].These compounds were analyzed using the same conditions as were used for the samples (Duarte et al., 2010).

2.5.2. Characterization of volatile compounds via HS-SPME/GC

2.5.2.1. Extraction of volatiles via headspace-SPME

The extraction of the volatile compounds was performed according Gonzalez-Rios et al. (2007), with some modifications. A divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) 50/30 µm SPME fiber (Supelco Co., Bellefonte, PA, USA) was used to extract the volatile constituents from the coffee headspace. Coffee samples (two grams) were macerated using nitrogen, placed into a 15 mL hermetically sealed flask, and heated for 10 min at 60 °C to reach sample headspace equilibrium. Then, the volatile compounds were extracted by placing the SPME fiber in the headspace for 30 min at 60 °C. For

compound desorption, the fiber was placed in the GC injection port and heated to 230 °C for 5 min.

2.5.2.2. HS-SPME/GC analysis

Analysis was performed using a gas chromatograph (GC), a Shimadzu model 17A equipped with a FID (flame ionization detector) and a capillary column of silica DB wax (30 m × 0.25 mm i.d. × 0.25 μm) (J&W Scientific, Folsom, Calif., U.S.A.). The oven temperature was maintained at 50 °C for 5 min. The temperature was then raised to 190 °C in increments of 3 °C/min and maintained at 190 °C for 10 min. The injector and detector temperatures were kept at 230 and 240 °C, respectively. The carrier gas (N_2) was maintained at a flow rate of 1.2 mL/min. The volatile compounds were identified by comparing the retention times of the compounds with those of standard compounds injected under the same conditions (Duarte et al., 2010).

The volatile compounds from each headspace analysis were defined by integrating the peak areas of all the identified compounds. The relative percentages of the individual compounds were calculated from

the total contents of the volatiles on the chromatograms (Petisca et al., 2013).

2.6. Analysis of sensory characteristics

The samples were prepared according to the Specialty Coffee Association of America (SCAA, 2013). The coffee was roasted in a laboratory roaster (Probatino, Leogap model, Brazil) with a capacity of 150 g and was then ground in an electric mill (Pinhalense ML-1, Brazil). A panel of three trained coffee tasters with Q-Grader Coffee Certificates was used for this study.

A panel of trained coffee experts used temporal dominance of sensations (TDS) analysis (Pineau et al., 2009) to identify the most relevant attributes for describing the temporal evolution of sensations for each sample. The attributes selected by the panel were as follows: chocolate, bitter chocolate, caramel, citric, tobacco, butter, herbaceous, and nuts. The analysis was performed on 7 sips. For each sip, the panelist imbibed the coffee, moved it around in their mouths for 3 s, and swallowed it. The evaluation continued until no sensation was perceived or 15 s had passed. The act of swallowing the sample facilitated the

temporal sensory perception of the product, according to the panelists' reports. Samples coded with three digits were submitted in a balanced order (Wakeling and MacFie, 1995) and evaluated in three replicates. The data were recorded using SENSOMAKER Software (Nunes and Pinheiro, 2012) and plotted as TDS curves showing the percentage of subjects who selected the attribute as dominant at a specific time, i.e., the dominance rate (Pineau et al., 2009).

3. RESULT

3.1. Quantification and characterization of microbial population

The bacteria and yeast populations found during coffee processing in two farms, Lavras (L) and Monte Carmelo (MC), are shown in Figure 1.

The yeast population at L farm ranged from 2.48 log cfu/g to 4.91 log cfu/g, and the smallest population (Fig. 1A) was observed at the beginning of drying. The yeast population in coffee from MC farm was lower than that in coffee from L farm, ranging from 2 log cfu/g to 4.85

log cfu/g. At the end of fermentation (28 h), it was not possible to detect the yeast population via the plating method (Fig. 1B).

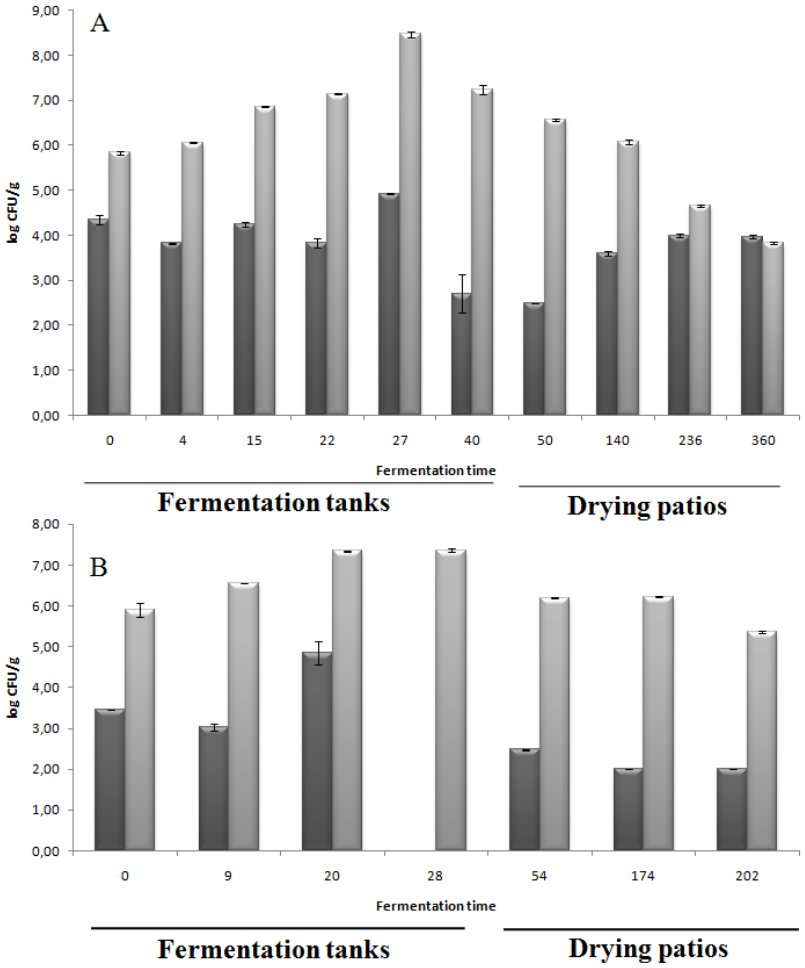

Fig. 1: The populations of yeast and bacteria in coffee wet processing at Lavras farm (A) and Monte Carmelo farm (B). Bacteria (▒) and Yeast (■).

The population of bacteria increased during wet fermentation and decreased during drying. The variation in the bacteria population was from 3.83 log cfu/g to 8.47 log cfu/g at L farm and from 5.37 log cfu/g to 7.36 log cfu/g in MC farm. The bacteria population was higher than the yeast population during all of fermentation in both coffee regions (Fig. 1).

A total of 251 isolates were obtained from samples of L farm; 59.36% of these isolates were yeasts, and 40.63% were bacteria (50.9% were Gram-positive, and 49.01% were Gram-negative). A total of 184 isolates were obtained from MC farm; 33.69% of these isolates were yeasts, and 66.3% were bacteria (33.6% were Gram-positive, and 66.3% were Gram-negative).

The isolates were grouped by phenotypic characteristics (cell morphology and biochemical features) (data not shown), and 236 selected isolates were subjected to rep-PCR for genotypic characterization. Representative isolates from each rep-PCR-profile were identified by sequencing, and a total of 27 bacterial and 16 yeast strains were identified (Table 1).

Table 1: List of identified eukaryote and prokaryotic microorganisms in coffee Lavras farm (L) and coffee Monte Carmelo farm (MC), present in grain coffee (G), during fermentation tank (T) and during drying (D).

Species identification on the basis of the highest similarity score	Region	Spot	Percentage of isolates (%) Lavras	Monte Carmelo
Yeast				
Hanseniaspora uvarum FJ231455.1 - 99.7%; KF953902.1 - 99%	L/MC	G/T/D	35.57	8.06
Pichia caribbica FN428941.1 - 100%; GU248264 - 99%	L/MC	G/T/D	53.02	12.90
Pichia fermentans DQ674358 - 100%	L/MC	G/T	5.37	3.23
Debaryomyces hansenii EF643588 - 100%	L	G/T	0.67	-
Torulaspora delbrueckii KF057608 - 100%; HE616749 - 99%	L/MC	G/T/D	0.67	50.00
Candida railenensis FM178302 - 100%	L	G	1.34	-
Candida quercitrusa KF747756 - 99%	L	T	0.67	-
Candida glabrata AY939793 - 99%	MC	G/T	-	6.45
Wickerhamomyces anomalus GQ376076 - 100%	MC	T	-	8.06
Wickerhamomyces ciferrii JQ901931.1 - 100%	L	D	0.67	-
Wickerhamomyces sp. JQ726611.1 - 98%	MC	T	-	3.23
Filamentous fungus				
Geotrichum candidum KF112070 - 99%	MC	G/T	-	3.23
Geotrichum citri-aurantii JQ425847 - 98%	L	G/T	1.34	-
Galactomyces pseudocandidum JN974292.1 - 99%	L	G/T	0.67	-
Galactomyces reessii HQ436459 - 99%	MC	G/T	-	1.61
Galactomyces sp. JQ437602.1 - 98%	MC	G/T	-	3.23

Table 1 (continued)

Bacteria				
Enterobacter asburiae JQ659696.1 - 100%; CP003026.1 - 99%	MC	G/T/D	-	41.80
Enterobacter ludwigii KC139450.1 - 100%	MC	G/T	-	11.48
Escherichia hermannii JX968501.1 - 100%	L	G	0.98	-
Enterococcus sp. KJ210577.1 - 99%	L	G/T/D	6.86	-
Ochrobactrum pseudogrignonense KF844052.1 - 100%	L	D	2.94	-
Chryseobacterium taichungense NR_042345.1 - 99%	L	G/T	10.78	-
Chryseobacterium bovis EF204448.1 - 100%	L	T	3.92	-
Chryseobacterium pallidum NR_042504.1 - 99%	MC	G	-	0.82
Erwinia persicina EU681952.1 - 100%	L	G/T/D	19.61	-
Pantoea sp. KF828863.1 - 90%	L	T	3.92	-
Pantoea agglomerans KC210845.1 - 99%	MC	T	-	0.82
Pantoea dispersa KF542916.1 100%; JQ659988.1 - 100%	MC	G/T	-	6.56
Staphylococcus warneri KF876878.1 - 100%	L	G/T	35.29	-
Pseudomonas plecoglossicida KF815701.1 - 100%	L	G	1.96	-
Paenibacillus amylolyticus AB695341.1 - 99%	L	D	0.98	-
Ochrobactrum pseudogrignonense KF844052.1 - 100%	L	D	2.94	-
Curtobacterium citreum FJ823008.1 - 99%	L	G/D	6.86-	-
Klebsiella oxytoca KC155255.1 - 100%	L/MC	T	4.90	3.28
Serratia marcescens JX103473.1 - 99%	MC	G	-	1.64
Microbacterium testaceum KC764967.1 - 99%	MC	T	-	1.64

Table 1 (continued)

Microbacterium sp. JQ660162.1 - 96%	MC	T	-	0.82
Microbacterium lacticum HQ292696.1 - 99%	MC	T	-	0.82
Microbacterium laevaniformans KF241157.1 - 100%	MC	T	-	1.64
Weissella cibaria AB911503.1 - 99%	MC	T	-	2.46
Bacillus amyloliquefaciens KF933607.1 - 100%	L	G	0.98	-
Kocuria sp. HM045839.1 - 99%	MC	T	-	1.64
Leuconostoc mesenteroides KF697635.1 - 99%	MC	G/T/D	-	24.59

The yeast species present during the fermentation process in coffee beans of L farm were *Hanseniaspora uvarum*, *Pichiacaribbica*, *P. fermentans, Debaryomyces hansenii, Torulaspora delbrueckii*, *Candida railenensis, Wickerhamomyces ciferria,* and *C. quercitrusa*, of which *P.caribbica* (53%) and *H. uvarum* (35.57%) were the predominant species (Table1). Some species of yeast identified in samples from MC farm were similar to those from L farm. However, *Hanseniaspora uvarum, Pichiacaribbica*, *P. fermentans, Torulaspora delbrueckii*, *Wickerhamomyces anomalus, Wickerhamomyces* sp., and *Candida glabrata* were identified only in MC farm (Table 1), and the species *Debaryomyces hansenii, C. railenensis, C. quercitrusa,* and *Wickerhamomyces ciferrii* were identified only in L farm. *Torulaspora delbrueckii* (50%) was the predominant yeast during the fermentation process in MC farm.

Some filamentous fungi were identified in YEPD medium in samples from L farm (*Geotrichum citri-aurantii* and *Galactomyces pseudocandidum*) and from MC farm *(Geotrichum candidum, Galactomyces reessii* and *Galactomyces* sp).

The bacteria population was more diverse than the yeast population (Table1). *Klebsiella oxytoca* was the only bacterial species found in both farms. *Escherichia hermannii*, *Enterococcus* sp., *Ochrobactrum pseudogrignonense*, *Chryseobacterium taichungense*, *Chryseobacterium bovis*, *Erwinia persicina, Pantoea* sp., *Staphylococcus warneri, Paenibacillus amylolyticus, Pseudomonas plecoglossicida, O. pseudogrignonense*, *Curtobacterium citreum*, and *Bacillus amyloliquefaciens* were only found in samples from L farm. The species *Staphylococcus warneri* (35.29%) and *Erwinia persicina* (19.61%) were the predominant bacteria during the fermentation process (Table 1). *Enterobacter asburiae, E. ludwigii, Chryseobacterium pallidum, P. agglomerans*, *Pantoea dispersa*, *Serratia marcescens, Microbacterium testaceum, Microbacterium* sp., *M. lacticum, M. laevaniformans*, *Weissella cibaria*, *Kocuria* sp., and *Leuconostoc mesenteroides* were the bacteria isolated from MC farm. The species *Enterobacter asburiae* (41.80%) and *Leuconostoc mesenteroides* (24.59%) were the predominant bacteria during the fermentation process (Table 1).

3.2 Culture-independent microbiological analysis using PCR-DGGE

Population dynamics were evaluated via PCR-DGGE in coffee cherries during fermentation and until the end of drying, as shown in Figures 2 and 3 and Table 2. The PCR-DGGE fingerprints demonstrated that the bacterial communities were more diverse than the yeast communities in all samples. In general, most species of bacteria present in the coffee bean before fermentation remained throughout the process and were also present in the water at the end of fermentation.

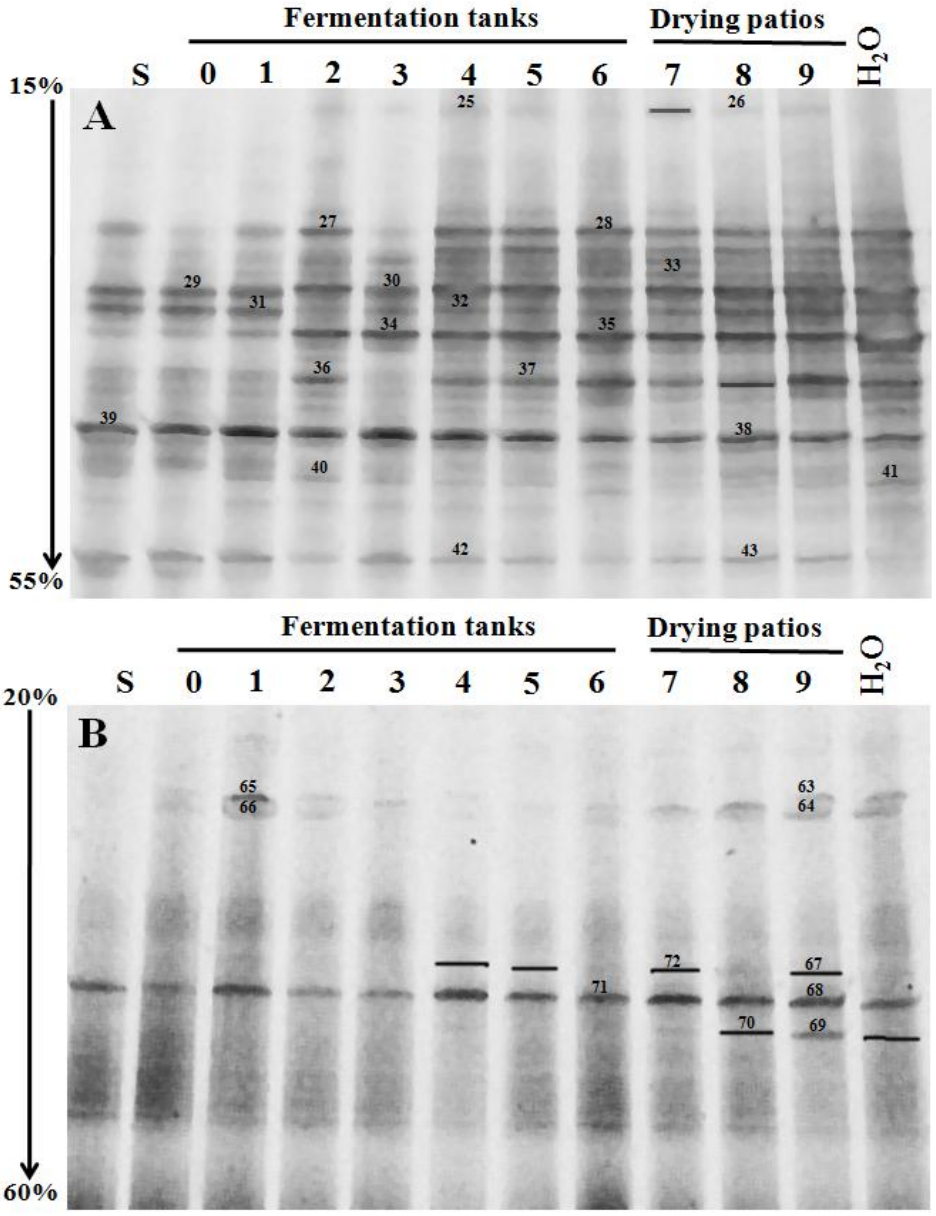

Fig. 2:PCR-DGGE patterns of the yeast and bacteria communities present during coffee wet processing at Lavras farm. Prokaryote (A) and Eukaryote (B).S = grain coffee, 0 = 0 hours, 1 = 4 hours, 2 = 15 hours, 3 = 22 hours, 6 = 27 hours, 7 = 40 hours, 8 = 50 hours, 6 = 140 hours, 7 = 236 hours, 8 = 360 hours, H_2O = water fermentation tank.

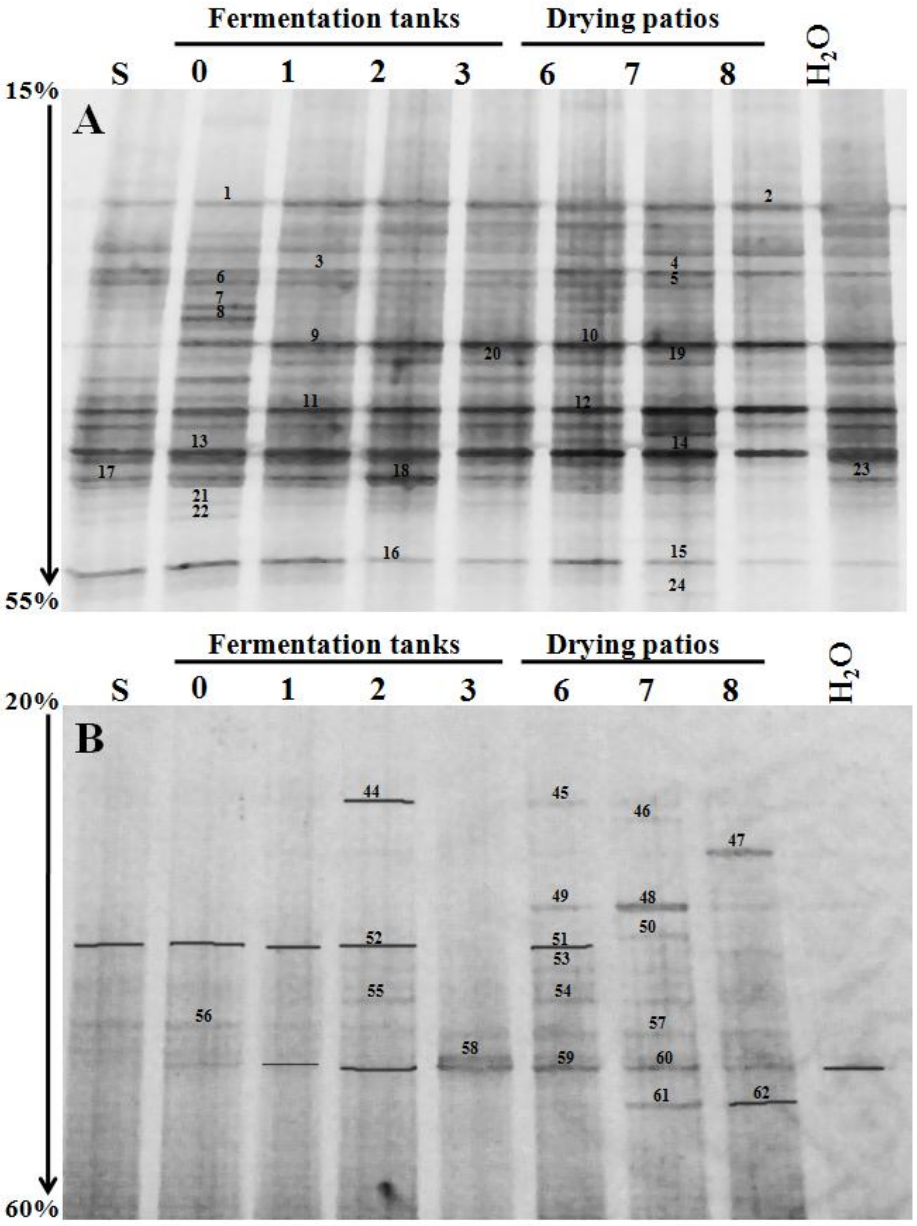

Fig. 3: PCR-DGGE patterns of the yeast and bacteria communities present during coffee wet processing at Monte Carmelo farm. Prokaryote (A) and Eukaryote (B).S = grain coffee, 0 = 0 hours, 1 = 9 hours, 2 = 20 hours, 3 = 28 hours, 6 = 54 hours, 7 = 174 hours, 8 = 202 hours, H_2O = water fermentation tank.

Table 2: Species identified via PCR-DGGE using universal primers for yeast and bacteria.

Bands	Acess number	Similarity (%)	Prokaryote
1	AF515228	95	*Leuconostoc pseudomesenteroides*
2,3, 27, 28,	AB854267	98	*Weissella confusa*
4, 5	HM756486	97	*Lysinibacillus fusiformis*
6	KF673524	97	*Lactobacillus fermentum*
7, 8, 11, 12, 15, 16	KF031439	100	Uncultured bacterium
9, 10	HQ683968	97	*Klebsiella oxytoca*
13,14	JX120129	98	*Actinobacterium* sp.
29, 30, 31, 32, 40, 41, 47, 48	FJ406528	95	Uncultured bacterium
33	JX315564	94	*Acinetobacter schindleri*
34, 35	HG798481	100	*Lactococcus lactis*
36, 37	KF453765	97	*Enterobacteriaceae bacterium*
38, 39	KF891342	97	*Enterobacter cloacae*
42, 43, 44	KC430956	95	*Enterobacter* sp.
45, 46	CP002272	97	*Enterobacter lignolyticus*
49	JX242698	94	Uncultured actinomycete

Table 2 (continued)

			Eukaryote
17, 18, 19, 20, 50, 51, 52	EU568995	95	*Pichia caribbica*
21, 22, 58, 59	JQ417238	98	*Mitchella repens*
23, 24, 60, 61, 62	KC510080	100	*Pichia fermentuns*
25, 26, 63, 64	AY314792	100	Uncultured fungus
53	EU650386	98	*Coffea arabica*
54, 55	AY520372	97	*Candida* sp.
56, 57	HE799671	97	*Torulaspora delbrueckii*

The sequencing of the bands of samples from L farm indicated the presence of *Leuconostoc pseudomesenteroides* (band 1), *Weissella confusa* (bands 2 and 3), *Lysinibacillus fusiformis* (bands 4 and 5), uncultured bacteria (bands 7, 8, 11, 12, 15, and 16), *Lactobacillus fermentum* (band 6), *Klebsiella oxytoca* (bands 9 and 10), *Actinobacterium* (bands 13 and 14) (Fig. 2A and Table 2). The sequencing of the bands of samples from MC farm indicated the presence of *Weissella confusa* (bands 27 and 28), uncultured bacteria (bands 29, 30, 31, 32, 40, 41, 47, and 48), *Acinetobacter schindleri* (band 33), *Lactococcus lactis* (bands 34 and 35), *Enterobacteriaceae bacterium* (band 36 and 37), *Enterobacter cloacae* (bands 38 and 39), *Enterobacter* sp. (bands 42, 43, and 44), *Enterobacter lignolyticus* (bands 45 and 46), and uncultured actinomycete (band 49) (Fig. 3A and Table 2). The yeast populations observed through PCR-DGGE in both experiments showed that the yeast species present in the coffee beans before being placed into the tank remained throughout the fermentation process (Figures 2B and 3B and Table 2). The sequencing of the bands of samples from MC and L farms indicated the presence of *Pichia caribbica* (bands 17, 18, 19, 20, 50, 51, and 52), *Candida* sp. (bands 54 and 55), *Torulaspora delbrueckii*

(bands 56 and 57), *Pichia fermentuns* (bands 23, 24, 60, 61, and 62), and uncultured fungi (bands 25, 26, 63, and 64). The plant species *Coffea arabica* (band 53) and *Mitchella repens* (bands 21, 22, 58 and 59) were also identified because the primers NL1 and LS2 are universal. Therefore, they can amplify the DNA of various eukaryotic organisms, including plants.

3.3 Chemical analyses

The main acid present in the coffee mucilage throughout the process of fermentation tank and drying was lactic acid (Fig 4), which showed increasing concentrations and reached a maximum value of 2.33 g/Kg in L farm, while in MC farm, the maximum concentration was 1.07 g/Kg. Other organic acids (citric, malic, and succinic acids) were also detected in lower concentrations in the coffee mucilage in both L farm and MC farm (Figure 4). Acetic acid was detected in the coffee mucilage only at the end of fermentation in both experiments. Propionic and butyric acids, which may impair the sensory characteristics of coffee, were not detected in the coffee mucilage.

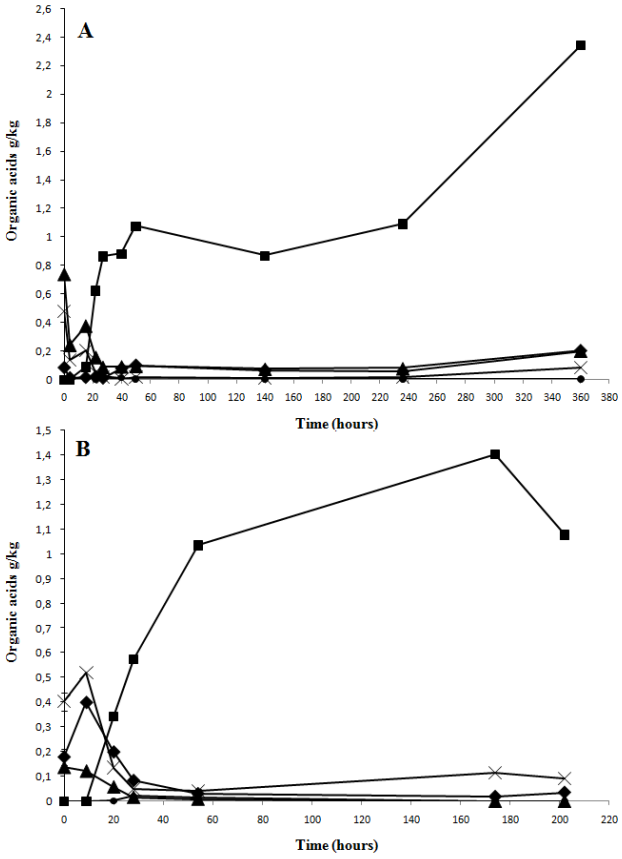

Fig. 4: Organic acids present in mucilage during coffee wet processing at Lavras (A) and Monte Carmelo (B) farms. Citric (◆), malic (▲), succinic (✕), lactic (■), acetic (●).

Sugars were evaluated, and the main sugar present in the coffee mucilage in both farms was fructose (3.18 g/Kg in coffee from L farm and 1.48 g/Kg for MC farm), followed by glucose (1.87 g/Kg in coffee from L farm and 0.68 g/Kg for MC farm) and sucrose (0.93 g/Kg in coffee from L farm and 0.60 g/Kg in coffee from MC farm).

The volatile compounds detected in the green and roast coffee are shown in Table 3. A total of 35 volatile compounds were detected via HS-SPME/GC. Among these compounds, 15 were detected in green coffee and 30 were detected in roasted coffee. The composition of green coffee beans for the two farms was different: the green coffee from L farm showed alcohol (methanol), esters (ethyl butyrate and furfuryl acetate), and acids (hexanoic and nonanoic acid) as the main compounds, while the green coffee from MC farm showed alcohol (methanol), acids (isobutyric and hexanoic acid), and furan (furfuryl alcohol and furfural). The composition of the majority of volatile compounds in roasted coffee was similar in both coffees studied, and the main compounds were furans (furfuryl alcohol and furfural), acids (isobutyric acid), and alcohol (1-pentanol and 2-heptanol).

Table 3: Relative percentage of volatile compounds identified in green and roasted coffee.

Compounds	Green (%)		Roasted(%)	
	Coffee L farm	Coffee MC farm	Coffee L farm	Coffee MC farm
Diether				
1.1-dietoxyethane	0.35	nd	0.03	0.03
Ketones				
2.3-butanedione	nd	nd	0.07	nd
2-nonanone	nd	nd	0.80	1.28
Verbenone	nd	nd	0.01	0.03
Total ketones	nd	nd	0.88	1.30
Alcohols				
Methanol	16.10	12.24	0.10	0.09
1-propanol	nd	1.17	nd	nd
1-pentanol	nd	nd	1.35	1.24
2-heptanol	nd	nd	1.83	2.11
3-methyl-1-pentanol	0.95	nd	0.55	0.67
1-hexanol	nd	1.79	nd	nd
a-Terpineol	1.03	nd	nd	nd
b-Citronellol	nd	nd	0.28	0.26
Geraniol	nd	nd	0.03	nd
2-phenylethanol	1.41	0.65	0.08	0.06
Total alcohols	19.49	15.85	4.22	4.44
Aldehydes				
Acetaldehyde	0.17	0.28	0.10	0.14
Hexanal	nd	nd	0.01	nd
Octanal	nd	nd	0.44	0.29
Nonanal	nd	nd	0.55	1.06
Butyraldehyde	0.91	nd	0.66	0.47
Decyl aldehyde	nd	nd	0.34	0.32
Total aldehydes	1.07	0.28	2.10	2.29

Table 3 (continued)

Acids				
Isobutyric acid	nd	3.11	8.15	7.45
Butyric acid	nd	nd	0.02	0.03
Hexanoic acid	1.44	2.36	0.11	0.09
Octanoic acid	nd	nd	0.04	0.06
Nonanoic acid	0.80	0.95	0.02	0.02
Propanoic acid	nd	nd	0.54	0.35
Total acids	2.23	6.42	8.87	8.00
Esters				
Propyl acetate	0.52	nd	0.61	0.76
Isobutyl acetate	nd	nd	nd	0.01
Ethyl butyrate	3.27	0.80	nd	nd
Ethyl octanoate	nd	nd	0.01	0.03
Diethyl malonate	nd	nd	0.28	0.21
Phenyl acetate	nd	nd	0.32	0.29
Furfuryl acetate	4.13	nd	2.38	2.72
Diethyl malate	nd	nd	0.03	0.01
Diethylsuccinate	nd	1.14	nd	nd
Total esters	7.92	1.95	3.63	4.03
Phenol				
Guaiacol	nd	nd	0.58	0.64
Furans				
Furfuryl alcohol	nd	2.72	21.93	19.03
Furfural	nd	2.70	13.26	13.90
5-methylfurfural	0.94	0.58	0.42	0.77
Total furans	0.94	5.99	35.61	33.70
Total GC area	7163	9870	288508	215900

nd= not detected

3.4 Sensorial analyze

The most relevant attributes of the coffee were selected in the previous analysis and subsequently evaluated via TDS analysis**.** The

sensations were perceived in five sips taken over the course of 15 seconds. The TDS curves are presented in Fig. 5. In the coffee from L farm, sips 1 and 4 had a variety of sensations, but none was dominant. Sip 3 had dominant herbaceous and citric sensations, and sips 2 and 5 had a dominant citric sensation (Fig. 5A). In the coffee from MC farm, every sip showed a dominant sensation. Sips 1 and 3 had a dominant citric sensation, sips 2 and 4 had a dominant herbaceous sensation, and sip 5 had a dominant nut sensation (Fig. 5B).

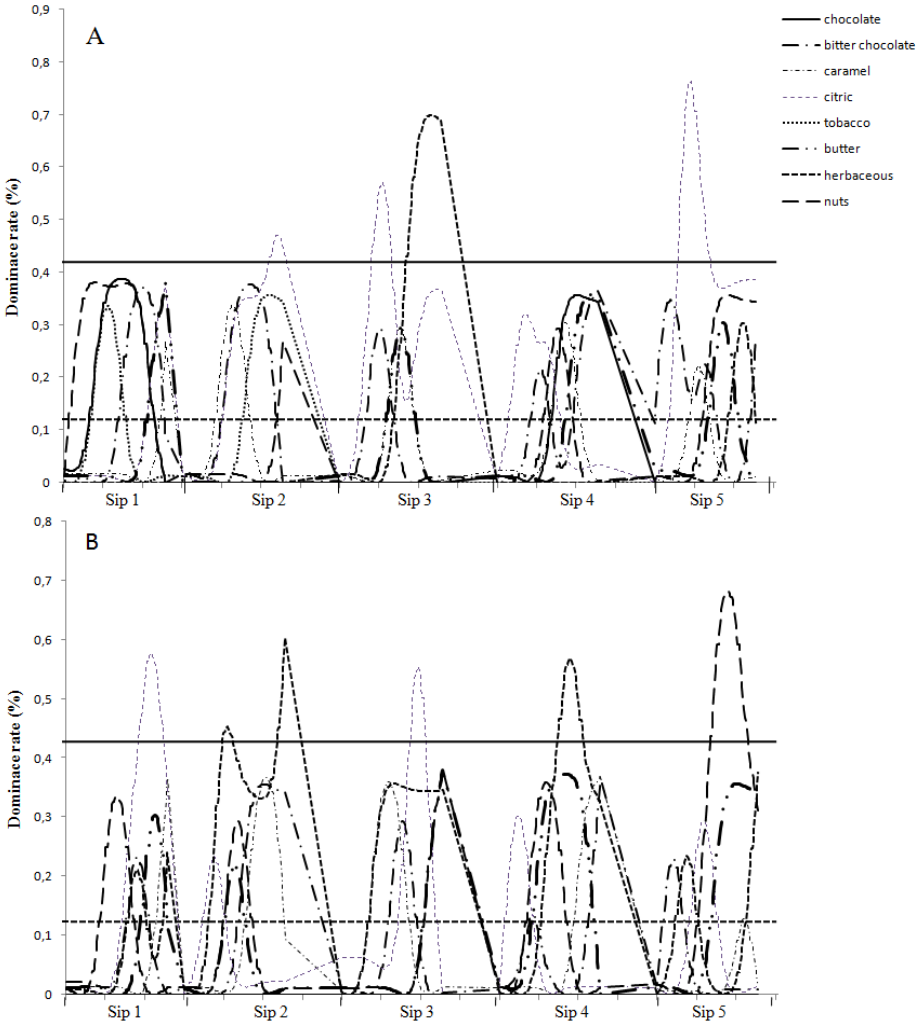

Fig. 5: TDS curves of coffee from L farm (A) and coffee from MC farm (B). Significance level (⁻) and chance level (¨).

Discussion

The variation in the microbial population was similar in the two regions studied (L farm and MC farm). The bacteria were dominant throughout the process and showed a high level of diversity (Figures 1, 2, and 3). In general, the bacteria population was larger than the yeast population, especially at the beginning of the fermentation, and these two groups of microorganism are the most commonly identified during coffee fermentation in Brazil when using the dry and semi-dry methods (Silva et al., 2000; Silva et al., 2008; Vilela et al., 2010).

A difference in the diversity of species of the prokaryotic and eukaryotic groups was observed (Table 1). This difference could have been due to several factors, such as environmental temperature, humidity, the composition of the remaining pulp and mucilage surrounding the coffee beans, and also the altitude (Long et al., 2014; Silva et al., 2008). Sixteen yeast species were identified, of which only four species were common to both regions: *Hanseniaspora uvarum*, *Pichia fermentans*, *Pichia caribbica*, and *Torulaspora delbrueckii*. The species *Hanseniaspora uvarum* and *Torulaspora delbrueckii* were detected in higher numbers (Table 1) in coffee from L farm and MC farm,

respectively, and were also reported in others studies that examined the wet and semi-dry processes (Masoud et al., 2004Vilela et al., 2010).

The presence of some yeasts, such as *Pichia* and *Hanseniaspora uvarum,* provides benefits for coffee processing due to their important role in the degradation of mucilage rich in pectin and because they may inhibit the growth of mycotoxigenic fungi (Masoud and Jespersen., 2006; Massawe et al., 2010; Silva et al., 2012). Yeasts of the genera *Saccharomyces*, *Candida,* and *Pichia* isolated during coffee processing might contribute to the degradation of coffee mucilage. Therefore, they are interesting strains that could be used as starter cultures (Evangelista et al., 2013; Silva et al., 2012). The genera *Geotrichum* and *Galactomyces* identified in this work showed morphological variation; these are filamentous fungi that may have a yeast-like colony morphology (Marcellino et al., 2001; Smith et al., 1995).

The greatest differences between the two coffee regions were observed in the bacteria species present. Twenty-six species were identified, but only *Klebsiella oxytoca* was detected in both coffee regions. *Staphylococcus warneri*, *Erwinia persicina*, and *Chryseobacterium taichungense* predominated in the coffee fermentation

at L farm, and *Enterobacter asburiae*, *Leuconostoc mesenteroides,* and *Enterobacter ludwigii* were the species predominating in samples from MC farm (Table 1). Some bacteria identified in this work belong to genera commonly found during coffee processing, such as *Erwinia*, *Klebsiella*, *Leuconostoc*, *Weissella*, *Enterococcus*, *Enterobacter*, *Serratia*, and *Bacillus*. These genera have been identified during the natural, semi-dry, and wet fermentation of coffee (Avalone et al., 2001; Leong et al., 2014, Silva et al., 2000; Silva et al., 2008; Vilela et al., 2010). Independent of the type of processing used, these genera may be present during the fermentation of coffee, but their population size may vary depending of type the processing used. For example, *Erwinia*, *Klebsiella*, and *Leuconostoc* are usually the main genera found during wet processing.

Some bacteria and yeasts that were present on coffee cherries persisted throughout fermentation and drying (Figures 2 and 3).Studies of the microbiota from coffee undergoing different types of processing showed that some yeasts and bacteria present during fermentation are already naturally present in the grain (Silva et al., 2000; Vilela et al., 2010).

Most species of yeast and bacteria found using the culture-dependent method were not detected via PCR-DGGE (Figures 2 and 3 and Table 2). Masoud et al. (2004) also reported that some microbial species were only detected using the culture-dependent method in samples taken during wet processing. This fact could be explained by the difficulty of obtaining high-quality DNA suitable for PCR directly from the samples. The initial template DNA and template competition may affect the detection of rare microorganisms in the microbial population (Muyzer et al., 1993).

Some bacterial species were detected only through PCR-DGGE, such as *Lactococcus lactis*, *Plautia stali*, *Lysinibacillus fusiformis*, *Lactobacillus fermentum*, and *Citrobacter freundii*. This may have occurred because their population densities were lower than 1 log CFU/g, making them impossible to detect via the culture-dependent method (Figures 2 and 3 and Table 2). Vilela et al. (2010) also reported that some microbial species were only detected using the DGGE method in samples taken during semi-dry processing. The results obtained in this work show that molecular methods should be used in conjunction with traditional methods to evaluate biodiversity.

At the beginning of fermentation, the sugar detected at the highest concentration was fructose, followed by glucose and sucrose. This analysis was realized in the mucilage surrounding the beans. This characteristic was also observed during a study of coffee wet processing in Mexico (Avallone et al., 2001), which also evaluated only the presence of sugars around the grain. The low amount of sucrose present in the mucilage in relation to fructose and glucose may be due to the action of invertase, which catalyzes the irreversible hydrolysis of sucrose to glucose and fructose. These enzymes may occur naturally in coffee fruit or be produced by microorganisms present during fermentation, such as *Pichiafermentans* (Geromel et al., 2008; Caputo et al., 2012).

Acetic and lactic acids were produced throughout the fermentation, but acetic acid was detected only at low concentrations. Lactic acid was the most abundant acid found in this study. The presence of mainly lactic acid bacteria, such as bacteria of the genera *Weissella*, *Leuconostoc*, and *Lactobacillus* (Leong et al., 2014; Schillinger et al., 2008), which were identified throughout the fermentation in this work, may have contributed to this fact.

The other acids detected were malic, citiric, and succinic acids (Fig. 4). These acids are already naturally present in the coffee bean, and their concentrations decreased during fermentation, but succinic acid showed a slight increase at the end of the process. Succinic acid may be produced by species of *Bacillus* spp (Silva et al., 2012) and by heterofermentative lactic acid bacteria (LAB) (Swiegers et al., 2005).

Volatile compounds were detected, including diether, ketones, alcohols, aldehydes, acids esters, phenol, and furans (Table 3). The compounds identified in green coffee beans differed between the regions studied (Table 3). One of the factors that may have influenced this difference is the microbiota involved during the processing of the coffee beans (Evangelista et al., 2013; Masoud et al., 2005; Silva et al., 2012). Some compounds are known to play a role in aroma development during fermentation, as esters that were detected in the green coffee beans in both regions.

However, after toasting, less difference in the volatile composition was observed (Table 3). The mechanisms of coffee aroma formation are extremely complex, and there is clearly a wide range of interactions between all the pathways involved. Maillard reactions occur between

reducing carbohydrates and proteins and are responsible for the formation of many volatile compounds during roasting (Akiyama et al., 2003; López-Galilea et al., 2006). Furans, phenolics, aldehydes, ketones, esters, alcohols, aldehydes, and other compounds are usually present in roasted coffee (Buffo and Cardelli-Freire, 2004).

The coffee from L farm presented a nice citrus flavor, and coffee from MC farm presented nut and herbaceous sensations as well. All these sensations are desirable in coffee. The presence of furans and ketones was detected in both samples, which contributed to the citric and herbaceous flavors that were dominant in the sensory analysis (Fig. 6). The presence of furans can provide herbal or fruity notes, and ketones are described as providing buttery, caramel-like, musty, mushroom like, or fruity notes (López-Galilea et al., 2006).

The evaluation of these two coffee-producing regions contributed to a better understanding of the microbiota present during coffee wet processing and some characteristics of this processing. In both regions, the coffee wet processing showed large populations of bacteria, and the main acid produced was lactic acid. The presence of volatile compounds differed between the regions and affected the beverages' final flavors.

The presence of different microbial species in each region may have contributed to this as well. In conclusion, this study evaluated coffee wet processing, which involved a variety of bacteria and yeasts, some of which prevailed during the fermentation. The combination of two techniques, culture-dependent and -independent methods, proved to be efficient in the understanding of the microbial population responsible for wet coffee fermentation. Future work should be conducted to evaluate the production of enzymes by the main species identified and select appropriate strains as starter cultures in coffee fermentation.

Acknowledgements

The authors thank the Brazilian agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico of Brasil (CNPQ), Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). We also thank the Juliana farm located in Monte Carmelo city and Resfriado farms situated in Lavras in the state of Minas Gerais, Brazil, for collecting samples.

References

Agate, A. D., Bhat J. V., 1966. Role of Pectinolytic Yeasts in the Degradationof Mucilage Layer of *Coffea robusta* Cherries. Applied Microbiology 14 (2), 256-260.

Akiyama, M., Murakami, K., Ohtani, N.,Iwatsuki, K., Sotoyama, K.,Wada, A., Tokuno, K.,Iwabuchi, H., Tanaka, K., 2003. Analysis of Volatile Compounds Released during the Grindingof Roasted Coffee Beans Using Solid-Phase Microextraction. Journal Agricultural Food Chemitry 51, 1961-1969.

Avallone, S., Guyot, B., Brillouet, J.-marc., Olguin, E., Guiraud, J.-pierre., 2001. Microbiological and Biochemical Study of Coffee Fermentation. Current Microbiology42, 252-256.

Caputo, L., Quintieri, L., Baruzzi, F., Borcakli, M., Morea M., 2012. Molecular and phenotypic characterization of *Pichia fermentans* strains found among Boza yeasts. Food Research International 48, 755-762.

Cocolin, L., Bisson, L.F., Mills, D.A., 2000. Direct profiling of the yeast dynamics in wine fermentations. FEMS Microbiology Letters 189, 81-87.

Buffo, R. A., Claudio Cardelli-Freire, C., 2004. Coffee flavour: an overview. Flavour and Fragrance Journal19, 99–104.

Duarte, W. F., Dias, D. R., Oliveira, J. M., Teixeira, J. A., Silva, J. B. A., Schwan, R. F., 2010. Characterization of different fruit wines made from cacao, cupuassu, gabiroba, jaboticaba and umbu. LWT - Food Science and Technology43(10), 1564-1572.

Durand, N., Sheikha, A. F., Suarez-Quiros M. L., Gonzales-Rios O., Nganou N. D., Fontana-Tachon A., Monte D., 2013. Application of PCR-DGGE to the study of dynamics and biodiversityof yeasts and potentially OTA producing fungi during coffee processing. Food Control 34, 466-471.

Devereux, R., & Willis, S. G.,1995. Amplification of ribosomal RNA sequences. In A D. L. Akkermans, J. D. Van Elsas, & F. J. Bruijn (Eds.), Molecular microbial ecology manual. Dordrecht: Kluwer Academic, pp. 1–11.

Evangelista, S. R., Silva, C. F., Miguel, M. G. P. C., Cordeiro, C. S., Pinheiro, A. C. M. Duarte, W. F., Schwan, R. F., 2013. Improvement of coffee beverage quality by using selected yeasts strains during the fermentation in dry process. Food Research International (in press).

Frank, H. A., Lum, N. A., Cruz,A. S. D., 1965. Bacteria Responsible for Mucilage-Layer Decompositionin Kona Coffee Cherries. Applied Microbiology 13 (2), 201 – 207.

Geromel, C., Ferreira, L. P., Davrieux, F., Guyot, B., Ribeyre, F., Scholz, M. B. S., Pereira, L. F. P., Vaast, P., Pot, D., Leroy T., Filho, A. A., Vieira, L. G. E., Mazzafera , P., Marraccini P., 2008. Effects of shade on the development and sugar metabolism of coffee (*Coffea arabica* L.) fruits. Plant Physiology and Biochemistry 46, 569-579.

Gonzalez-Rios, O., Suarez-Quiroza, M. L., Boulanger, R., Barel, M., Guyot, B.,. Guiraud, J. P., Schorr-Galindo, S., 2007. Impact of ‘‘ecological’’ post-harvest processing on coffee aroma: II. Roasted coffee. Journal of Food Composition and Analysis 20, 297–307.

Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T., Williams, S.T., 1994. Bergey's manual ofdeterminative bacteriology, ninth ed. W. & Wilkins, Baltimore.

Kurtzman, C.P., Fell, J.W., Boekhout, T., 2011. The Yeasts, a Taxonomic Study, fourth ed.Elsevier, Amsterdan.

Leong, K., Chen,Y., Pan, S., Chen, J., Wu, H., Chang, Y., Yanagida,F., 2014. Diversity of Lactic Acid Bacteria Associated with Fresh CoffeeCherries in Taiwan. Curr Microbiol 68, 440–447.

López-Galilea, I., Fournier, N., Cid, C., Guichard, E., 2006. Changes in Headspace Volatile Concentrations of Coffee BrewsCaused by the Roasting Process and the Brewing Procedure. Journal Agricultural Food Chemitry 54, 8560-8566.

Marcellino, N., Beuvier, E., Grappin, R., Guéguen, M., Benson, D. R., 2001. Diversity of *Geotrichum candidum*Strains Isolated from Traditional Cheesemaking Fabrications in France. Applied and Environmental Microbiology 67(10), p. 4752–4759.

Masoud, W., Cesar, L. B., Jespersen, L., Jakobsen, M., 2004. Yeast involved in fermentation of *Coffea arabica* in East Africa determined by genotyping and by direct denaturating gradient gel electrophoresis. Yeast 21, 549–556.

Masoud, W., Jespersen, L., 2006. Pectin degrading enzymes in yeasts involved in fermentation of *Coffea arabica* in East Africa. International Journal of Food Microbiology 110, 291–296.

Masoud, W., Poll L., Jakobsen M., 2005. Influence of volatile compounds produced by yeastspredominant during processing of Coffea arabica inEast Africa on growth and ochratoxin A (OTA)production by *Aspergillus ochraceus.* Yeast 22, 1133–1142.

Massawe, G. A., Lifa, S. J., 2010. Yeasts and lactic acid bacteria coffee fermentation starter cultures. International Journal of Postharvest Technology and Innovation2(1), 41-80.

Ministério da Agricultura, Pecuária e Abastecimento (MAPA) (2014). Available in: <http://www.agricultura.gov.br/>Accessed at: Jan. 2014.

Muyzer, G., Waal, E. C., Uitterlinden, A. G., 1993. Profile of complex microbialpopulations by denaturing gradient gel electrophoresis analysis of polymerasechain reaction: Amplified genes coding for 16S rDNA. Applied and Environmental Microbiology 59, 695–700.

Nielsen, D.S., Teniola, O.D., Ban-Koffi, L., Owusu, M., Andersson, T.S., Holzapfel, W.H.,2007. The microbiology of Ghanaian cocoa fermentations analysed using culturedependentand culture independent methods. International Jounal of Food Microbiology114, 168–186.

Nunes, C. A.; Pinheiro, A. C. M., 2012. Sensomaker Free user-friendly interface for sensory analysis. Available from: www.ufla.br/sensomaker (Accessed at: Out. 2012).

Ovreas, L., Forney, L., Daae, F. L., 1997. Distribution of Bacterioplankton in Meromictic Lake Saelenvannet, as Determined by Denaturing Gradient Gel Electrophoresis of PCR-Amplified Gene Fragments Coding for 16S rRNA. Applied and Environmental Microbiology 63(9), 3367–3373

Oliveira, P. D.; Borém, F. M.; Isquierdo, E. P.; Giomo, G. S.; Lima, R. R.;. Cardoso, R. A.; 2013. Aspectos fisiológicos de grãos de café, processados e secados de diferentes métodos, associados à qualidade sensorial. Coffee Science 8 (2), 211-220.

Parkouda, C., Thorsen, L., Compaoré, C.S., Nielsen, D.S., Tano-Debrah, K., Jensen, J.S.,Diawara, B., Jakobsen, M., 2010. Microorganisms associated with Maari,a Baobab seed fermented product. International Journal of Food Microbiology142, 292-301.

Pineau, N., Schlich, P., Cordelle, S., Mathonnière, C., Issanchou, S., Imbert, A., Rogeaux, M., Etiévant, P., Koster, E., 2009. Temporal Dominance of Sensations: Construction of the TDS curves and comparison with time–intensity. Food Quality and Preference 20, 450-455.

Petisca, C., Pérez-Palacios, T., Farah, A., Pinho, O., Ferreira, I. M. P. L. V. O., 2013. Furans and other volatile compounds in ground roasted and espresso coffee using headspace solid-phase microextraction: Effect of roasting speed. Food and Bioproducts Processing 91(3), 233-241.

Ramos, C. L., de Almeida, E. G., Pereira, G. V. D. M., Cardoso, P. G., Dias, E. S., Schwan, R. F., 2010. Determination of dynamic characteristics of microbiota in a fermented beverage produced by Brazilian Amerindians using culture-dependent and culture-independent methods. International Journal of Food Microbiology1 40(2-3), 225-31.

Schillinger, U., Boehringer, B., Wallbaum, S., Caroline, L., Gonfa, A., Huch M., Holzapfel, W. H.,. Franz,C. M.A.P., 2008.Agenus-specic PCRmethod for differentiation between*Leuconostoc* and*Weissella*and its application in identication ofheterofermentative lactic acid bacteria from coffee fermentation. FEMS Microbiol Lett 286, 222–226.

Schwan, R. F., Silva C. F., Batista L. R., 2012. Coffee fermentation. In: Hui, Y. H . (Ed), Handbook of plant-based fermented food and beverage technology.CRC Press, Boca Raton, pp. 677–690.

Specialty Coffee Association of America (SCAA) (2013). Available in: <http://www.scaa.org> Accessed at: April. 2013.

Senguna, I. Y., Nielsen, D. S., Karapinar, M., & Jakobsen, M., 2009. Identification of lactic acid bacteria isolated from Tarhana, a traditional Turkish fermented food. International Journal of Food Microbiology 135, 105–111.

Silva, C. F., Batista, L. R., Abreu, L. M., Dias, E. S., & Schwan, R. F., 2008. Succession of bacterial and fungal communities during natural coffee (C*offea arabica*) fermentation.Food Microbiology 25(8), 951-7.

Silva, C F, Schwan, R. F., Dias, E. S., Wheals, A. E., 2000. Microbial diversity during maturation and natural processing of coffee cherries of Coffea arabica in Brazil. International Journal of Food Microbiology 60(2-3), 251-60.

Silva, C. F., Vilela, D. M., Souza, C. C., Duarte, W. F., Dias, D. R., Schwan, R. F., 2012. Evaluation of a potential starter culture for enhance quality of coffee fermentation. World Journal of Microbiology & Biotechnology 29(2), 235-47.

Sindicato da Indústria de Café do Estado de Minas Gerais (Sindcafé-MG) (2014). Available in: <http://sindicafe-mg.com.br/plus/modulos/conteudo/?tac=tipos-de-cafe>Accessed at: Jan. 2014.

Smith, M. T., Cock, A. W. A. M., Poot, G. A.,. Steensma, H. Y.,1995. Genome Comparisons in the Yeastlike FungalGenus *Galactomyces* Redhead et Malloch. International Journal of Systematic Bacteriology 45(4), p. 826–831.

Swiegers, J. H., Bartowsky, E. J., Henschke, P. A., & Pretorius, I. S., 2005. Yeast andbacterial modulation of wine aroma and flavor. Australian Journal of Grape and Wine Research 11, 139–173.

Vilela, D. M., Pereira, G. V. D. M., Silva, C. F., Batista, L. R., Schwan, R. F., 2010. Molecular ecology and polyphasic characterization of the microbiota associated with semi-dry processed coffee (*Coffea arabica L.*). Food Microbiology 27(8), 1128-35.

Wakeling, I. N., Macfie, H. J. H., 1995. Designing consumer trials balanced for first and higher orders of carry-over effect when only a subset of k samples from t may be tested. Food Quality and Preference 6, 299-308.