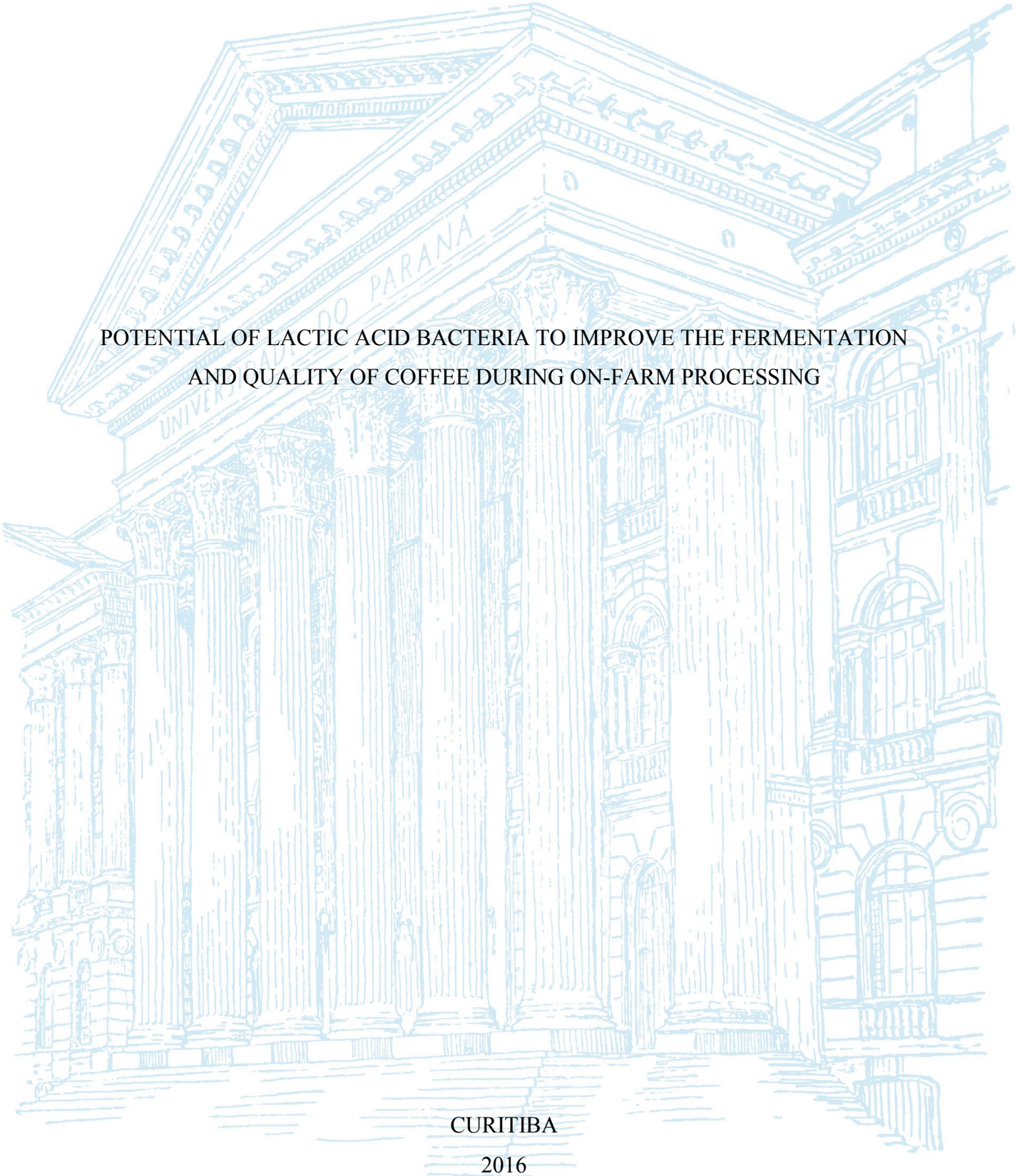
UNIVERSIDADE FEDERAL DO PARANÁ

DÃO PEDRO DE CARVALHO NETO



POTENTIAL OF LACTIC ACID BACTERIA TO IMPROVE THE FERMENTATION AND QUALITY OF COFFEE DURING ON-FARM PROCESSING

CURITIBA

2016

DÃO PEDRO DE CARVALHO NETO

POTENTIAL OF LACTIC ACID BACTERIA TO IMPROVE THE FERMENTATION AND QUALITY OF COFFEE DURING ON-FARM PROCESSING

Dissertação apresentada como requisito parcial à obtenção do grau de Mestre em Engenharia de Bioprocessos e Biotecnologia, no Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, Setor de Tecnologia da Universidade Federal do Paraná.

Orientador: Prof. Dr. Gilberto Vinícius de Melo Pereira
Co-Orientador: Prof. Dr. Carlos Ricardo Soccol

CURITIBA

2016

Catalogação na Fonte: Sistema de Bibliotecas, UFPR
Biblioteca de Ciência e Tecnologia

C331l Carvalho Neto, Dão Pedro de
Potential of lactic acid bacteria to improve the fermentation and quality of coffee during on-farm processing / Dão Pedro de Carvalho Neto. – Curitiba, 2016.
32 f. : il. color. ; 30 cm.

Dissertação - Universidade Federal do Paraná, Setor de Tecnologia, Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, 2016.

Orientador: Gilberto Vinícius de Melo Pereira – Co-orientador: Carlos Ricardo Soccol,
Bibliografia: p. 28-30.

1. Café. 2. Bactérias. 3. Lactobacillus plantarum. I. Universidade Federal do Paraná. II.Pereira, Gilberto Vinícius de Melo. III. Soccol, Carlos Ricardo .
IV. Título.

CDD: 663.93

Bibliotecário: Elias Barbosa da Silva CRB-9/1894

Ministério da Educação
Universidade Federal do Paraná
Pró Reitoria de Pesquisa e Pós Graduação
Setor de Tecnologia
Programa Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia

TERMO DE APROVAÇÃO

Os membros da Banca examinadora designada pelo Colegiado do Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia da Universidade Federal do Paraná, foram convocados para realizar a arguição da Dissertação de Mestrado de Dão Pedro de Carvalho Neto intitulada: **"Potential of lactic acid bacteria to improve the fermentation and quality of coffee during on-farm processing"**, após terem inquirido o aluno e realizado a avaliação do trabalho são de parecer pela sua aprovação no rito de defesa. A outorga do título de mestre está sujeita à homologação do Colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e a pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

Curitiba, 29 de Setembro de 2016.

Prof. Dr. Gilberto Vinicius de Melo Pereira
Presidente da Banca Examinadora (UFPR)

Prof. Dr. Craig Faulds
Avaliador Externo (AMU)
Vídeo conferência

Prof. Dr. Carlos Ricardo Soccol
Avaliador Interno (UFPR)

EXTRATO
Ata da 65ª Reunião do Colegiado do Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia

Aos vinte e nove dias do mês de Agosto do ano de dois mil e dezessete, reuniram-se os membros do Colegiado do Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia (PPGEBB). Estavam presentes os professores: Júlio César de Carvalho, Luciana Porto de Souza Vandenberghe, Adriane Bianchi Pedroni Medeiros, Adenise Lorenci Woiciechowski, Carlos Ricardo Soccol, os professores convidados do DEBB Luiz Augusto Junior Letti, Cristine Rodrigues e Susan Grace Karp. A Professora Vanete Thomaz Soccol justificou sua ausência. Sob a presidência do professor Júlio César de Carvalho, coordenador do PPGEBB, que agradeceu a presença de todos, foi declarada aberta a sessão.3)Homologar Ata de aprovação dos alunos Raquel Khoeler Sanson, Dão Pedro de Carvalho Neto, Marcela Candido Camara e Giovanni Razera devida a falta de assinatura dos membros externos da França na Ata (videoconferência) – Foi aprovado por unanimidade pelo Colegiado.

Prof. Dr. Júlio César de Carvalho
Coordenador do PPGEBB

Marta Helena Szadkoski
Secretária Executiva do PPGEBB

Dedico esse trabalho aos meus pais, Wilson e Rosemeire, por todo o apoio, compreensão, presença e dedicação durante todas as etapas e desafios que vivenciei.

AGRADECIMENTOS

Ao meu orientador, Prof. Dr. Gilberto Vinícius de Melo Pereira, pela orientação, companheirismo e ensinamentos.

Ao Curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, do Setor de Tecnologia da Universidade Federal do Paraná, na pessoa do seu coordenador Prof. Dr. Carlos Ricardo Soccol, pelo apoio e oportunidades recebidas.

À Faculté des Sciences, da Aix Marseille Université, na pessoa do seu coordenador Prof. Dr. Craig Faulds pela grande oportunidade e aprendizado com a experiência do curso BioDEV.

Às Professoras Drª. Adriane Bianchi Pedroni Medeiros e Drª. Vanete Thomaz Soccol por todo apoio e suporte durante a condução do trabalho realizado.

Aos companheiros dos Laboratórios de Processos Biotecnológicos (LPB) pelo companheirismo e amizade durante toda a longa jornada.

PRESENTATION

The following work was carried out at Universidade Federal do Paraná (UFPR) in Bioprocess Engineering and Biotechnology department under the supervision of Pr. Carlos Ricardo Soccol, Ph.D., in conjunction with the Aix Marseille Université (AMU) under the supervision of Pr. Craig Faulds, Ph.D.

The Microbial Biotechnology for Sustainable Development (BIODEV) course was established in 2004 at the Ecole supérieure d'Ingénieurs de Marseille (ESIM) and attached to the Chair UNESCO in innovation for sustainable development. This program aims at training professionals able to achieve synergy between research and sustainable development applicable to the industry.

RESUMO

O presente estudo descreve o potencial uso de bactéria ácido lática na condução de fermentação de grãos de café durante o processamento úmido conduzido em condições de campo. Dentre as diversas cepas testadas, a *Lactobacillus plantarum* LPBR01 demonstrou uma produção desejada de ácidos orgânicos e ésteres aromáticos em um meio simulativo de polpa de café. A cepa foi capaz de estabelecer um processo acelerado de acidificação, potencialmente reduzindo o tempo de fermentação. A cepa também elevou significativamente a formação de compostos orgânicos voláteis, permitiu a produção de uma bebida com notas sensoriais distintas e estimulou uma maior diversidade de leveduras durante a fermentação. Os resultados foram parcialmente publicados na revista Internationa Journal of Food Science and Technology.

Palavras-chave: café, bactéria ácido lática, processamento via úmida, *Lactobacillus plantarum*

ABSTRACT

This study describes the potential use of lactic acid bacteria to conduct improved coffee bean fermentation during on-farm wet processing. Among different strains tested, *Lactobacillus plantarum* LPBBR01 showed a suitable production of organic acids and flavor-active esters in a coffee-pulp simulation medium. It was able to establish an accelerated acidification process, potentially reducing the fermentation time. The strain also increased significantly the formation of volatile aroma compounds, enabled the production of a beverage with distinct sensory notes and stimulate greater diversity of yeasts during the fermentation. The results were partially published at International Journal of Food Science and Technology.

Key-words: coffee, lactic acid bacteria, wet processing, *Lactobacillus plantarum*

LISTA DE FIGURAS

LISTA DE TABELAS

SUMÁRIO

1. INTRODUCTION

1.1. Coffee production and processing

Coffee is the non-alcoholic beverage most appreciated and consumed around the world, with a global production above 8.5 million tons in the 2014/15 crop (ICO, 2016). Although the *Coffea* genus comprises over 100 species, only *C. arabica* (Arabica coffee) and *C. canephora* (Robusta coffee) are grown worldwide for the production of coffee, accounting for 56 and 44% of the global production, respectively (CAGLIANI et al., 2013). With an annual output of 2.72 million tons, Brazil is the main producer and exporter of coffee beans, followed by Vietnam, Colombia, Indonesia, Ethiopia, India and Honduras.

The post-harvest processing consists, in general, on the removal of the pericarp and drying of the beans to a moisture content of approximately 10-12% fresh weight (JOËT et al., 2010). There are three methods used: the wet, dry and semi-dry processing. In wet processing (method used in this study), the coffee fruits are mechanically depulped (total and partial removal of the exocarp and mesocarp, respectively). Then, the berries are submerged in a water tank and subjected to a microbial fermentation for 24-48 hours where the remaining mucilage is degraded. Finally, the beans are sun-dried for 5-10 days until they reach a humidity of 10-12% (SILVA et al., 2000; BATISTA et al., 2009). This method provides a reduction in time and area required for drying the coffee beans.

The coffee beverage flavor and quality varies enormously and it is influenced by numerous factors, such as genetic strain of coffee tree, geographical region, climate, presence of defective beans (e.g. damaged by insects, those that are overmature) and the post-harvest processing method employed (OLIVEIRA et al., 2006; SUNARHARUM; WILLIAMS; SMYTH, 2014). It was observed that the same variety of coffee, when subjected to different processing method, presented distinct sensory profiles (SELMAR; BYTOF; KNOPP, 2002; ARRUDA et al., 2012). However, slight attention is given to this interrelation between the final cup quality and the fermentation associated to the processing method used, since the purpose of the latter is thought to be solely the removal of mucilage.

1.2. Lactic acid bacteria in coffee fermentation

The coffee fermentation references to the succession of a consortium of bacteria, yeasts and filamentous fungi already present in the coffee cherries (SILVA et al., 2008). The species of homo- and heterofermentative lactic acid bacteria (LAB) are prevalent in the early stages of fermentation, expressing 80% of the total of microorganisms detected (SILVA et al., 2008). These microorganisms are responsible for the initial conversion of reducing sugars in secondary metabolites (*i.e.,* lactic acid, acetaldehyde, ethanol). Species of *Leuconostoc* spp. (such as *I. mesenteroides*, *L. pseudomesenteroides*, *L. citreum*, *L. holzapfelli*), *Weissela* spp. (such as *W. cibaria, W. confused, W. thailandensis*) and *Lactobacillus* spp. (such as *L.brevis L. plantarum, L. lactis* subsp. *lactis*) were predominant in several studies involving isolation of microorganisms from coffee fruits and different types of processing (AVALLONE et al., 2001; DE BRUYNE et al., 2007; VILELA et al., 2010; VELMOUROUGANE, 2013; LEONG et al., 2014;).

Although present in significant amounts ($\geq 10^4$ $CFU.g^{-1}$), the role played by LAB is still unknown. The main functions associated with these microorganisms are: antifungal activity, capability to reduce the pH of the fermenting pulp-mass beans through organic acids production, prevention of the growth of spoilage bacteria and promotion of a selective and prone environment for yeast growth (HUCH; FRANZ, 2015; PEREIRA et al., 2015b).

1.3. Employment of starter cultures on coffee fermentation

Aroma and taste are the aspects with the greatest impact on the quality of coffee beverage. Over the years, it has been shown that driving a controlled fermentation would allow to the reduction of costs of process and enhance quality of end products (STEINKRAUS, 2004). Nevertheless, the development of a starter culture requires an extensive work comprising the isolation and identification of strains capable of efficiently convert the nutrients initially willing, inhibit food-related pathogenic microorganisms, preserve the final product, improve the sensory properties and promote the standardization and reproducibility of the process (TAMIME, 2003; PEREIRA et al., 2015b).

Although previous studies on the inoculation of microorganisms for coffee processing has been made in the mid-20th century (CALLE, 1957; BUTTY, 1973), it

was only in 2000's that such surveys were taken up and won greater support. Avallone et al. (2002) evaluated the physicochemical and microbiological characteristics of natural semi-dry fermentations by inoculating strains of pectinolytic bacterias and yeasts (viz. *Lactobacillus brevis* L166, *Erwina herbícola* C26, *Bacillus subtilis* C12 and *Kluyveromyces fagilis* K211). The authors observed that the inoculation has not elevated the population of the pectinolytic microflora and the enzymes showed no capability to depolymerize pectin, indicating that the removal of the mucilaginous layer is associated with acidification. However, the organoleptic characteristics remained unaltered, indicating that the microbiological control could reduce the development of off-flavors in the final beverage.

Velmourougane et al. (2011) assessed the control of Arabica and Robusta coffee fermentations regarding contamination by *Aspergillus ochraceus*, presence of OTA and cup quality when inoculated with commercial bakery yeast (*Saccharomyces cerevisae*). Upon inoculation to 2% yeast, the authors observed a reduction of 70.2 and 96% (Arabica) and 34.6 and 66.7% (Robusta) on the total contamination by *A. ochraceus* and total OTA concentration (ppb), respectively. Inoculation have not affected the final cup quality in both Arabica and Robusta coffees, making the *S. cerevisiae* a potential tool for biocontrol of OTA-producing fungi.

Pereira et al. (2015) evaluated the performance of aromatic yeast strain, *Pichia fermentans* YC5.2, in relation to growth and dominance, adaptability to coffee fermentation conditions and production of a higher quality beverage. On-farm spontaneous (control), inoculated and supplemented fermentations with 2% sucrose were conducted. Inoculated and supplemented fermentations showed a yeast count (3 log larger) and a frequency of *P. fermentans* five times higher compared to the control. In this study, all prepared coffee beverages were scored over 87 points, which indicates a very high coffee quality according to Specialty Coffee Association of America Cupping Protocol. However, remarkable sensory differences were observed due to increased production of yeast-derived metabolites (*i.e.,*acetaldehyde, ethyl acetate and isoamyl acetate), demonstrating that the inoculated strain had a significant impact on the sensory profile of the final beverage.

2. PRACTICAL OBJECTIVES

2.1. Main objective

The aim of this study was to select a LAB starter culture for controlled coffee bean fermentation and to determine its effects on fermentation performance and beverage quality.

2.2. Secondary objectives

- Selection of a LAB strain capable of producing desirable volatile compounds associated with coffee beverage quality (*i.e.,* ethyl acetate, ethyl isobutyrate, isoamyl acetate);
- Selection of a LAB strain with low production of undesirable metabolites (*i.e.,* acetic, butyric and propionic acids) that damage the final quality of coffee beverage;
- Establishing a methodology for on-farm implementation of LAB starter culture for coffee bean processing;
- Evaluate the effect of LAB inoculation on yeasts diversity during coffee fermentation process;
- Optimize the coffee bean fermentation process through faster acidification;
- Obtainment of a superior coffee beverage compared with the conventional process.

3. MATERIAL AND METHODS

3.1. Lactic acid bacteria screening

LAB strains used in this study (*viz., Lactobacillus plantarum* LPBL01, *Lactobacillus brevis* LPBL02, *Lactobacillus brevis* LPBL03, *Lactobacillus paracasei* ssp. *paracasei* LPBL07, *Lactobacillus* sp. LPBL08, *Lactobacillus* sp. LPBL10, *Lactobacillus* sp. LPBL14, *Lactobacillus plantarum* LPBR01, *Lactobacillus brevis* LPBR02, *Lactobacillus* sp. LPBR03, *Lactobacillus* sp. LPBR06, *Lactobacillus* sp. LPBR07 and *Lactobacillus* sp. LPBR08) were obtained from the culture collection of the Laboratory of Biotechnological Processes at LPBII/UFPR, Curitiba, Paraná State, Brazil. The LAB strains were evaluated for their ability to produce organic acids and volatile aromatic compounds in a coffee-pulp simulation medium (PEREIRA et al., 2014).

3.2. On-farm fermentation experiment

3.2.1. Microorganism and lyophilized inoculum preparation

The pre-culture was prepared by inoculating 100 mL of YEPG broth with 1 mL of the thawed stock culture of selected *Lactobacillus plantarum* LPBR01 strain and incubating for 48 h at 30 °C and 120 rpm. The LAB cells were then transferred to larger volumes until it reached a concentration of 10^{10} cells/mL. Lyophilization was carried out by resuspending the cells in skimmed UHT milk, rapidly freezing in an ethanol–dry ice mixture and freeze-drying in a Modulyod Freeze Dryer 230 (Thermo Electron Corporation, Waltham, USA) under negative pressure of 50 mBar at −45 °C.

3.2.2. Inoculation

The fermentation experiments were performed at the Fazenda Shalon, Minas Gerais state, Brazil. Freshly coffee beans were mechanically depulped (BDSV-04 Pinhalense depulper) to obtain beans with mucilage. Fermentations were conducted in cement tanks with a useful volume of 4.5 m³ containing 20 kg of depulped cherries and 500 L of fresh water, following the guidelines of the local wet processing method. Lyophilized starter culture was rehydrated by adding water at 37– 40 °C (10 g L^{-1}) and stirred gently over a period of 5 min. This solution was spread into the fermentation tank to reach a concentration of 10^7 cells mL^{-1}. As a control, spontaneous process was

allowed to ferment with indigenous microorganisms present in the coffee fruit. The fermentations were conducted in triplicate.

3.2.3. Sampling and pH

Samples (liquid and grain fraction) were collected in triplicate at 0 and 24 h for the total microbiological count and target metabolites analysis. The pH of the fermenting mass was measured at each sampling point using a portable pH meter, model AK90 (AKSO, São Leopoldo, Brazil).

3.3. Microbiological analysis

3.3.1. Enumeration of microorganisms

Samples (10 mL) were homogenized in 90 mL saline–peptone water in a Stomacher at normal speed for 5 min (10^{-1} dilution) and diluted serially. DRBC containing 100 mgL^{-1} chloramphenicol (Sigma) and MRS agar medium (Himedia) were used to account for total yeast and LAB, respectively. Plates were incubated at 30 °C for 48 h.

3.3.2. Isolation and identification of yeast

Yeasts were isolated from the DRBC agar plates. The isolates were grouped and identified by molecular techniques. The grouping was performed using the repetitive sequence based polymerase chain reaction (rep-PCR) technique, being evaluated the fingerprint generated for each microorganism. Identification was performed by sequencing of ITS4 region. The sequences obtained were compared with sequences available in the GenBank database through a basic local alignment search tool (BLAST).

3.4. High-Performance Liquid Chromatography analyses

Concentrations of reducing sugars (glucose and fructose) and organic acids (lactic, acetic, citric, fumaric, succinic and malic acids) of the fermenting coffee-pulp and of the coffee-pulp simulation medium were analyzed by high-performance liquid chromatography (HPLC).

3.5. Gas Chromatography–SPME analysis

A carboxen/poly (dimethylsiloxane) (DVB/CAR/PDMS) type 75 μm Solid Phase Microextraction (SPME) fiber (Supelco Co., Bellefonte, PA, USA) was used to extract volatile constituents from the headspace of the fermenting coffee pulp–bean mass and coffee pulp simulation medium fermentation. The sample was prepared by diluting two milliliters in 1:1 ratio with distilled water plus NaCl 5% (w/v). The standards used were:

- 12 esters (ethyl acetate, propyl acetate, ethyl laurate, ethyl propionate, ethyl isobutyrate, ethyl hexanoate, ethyl octanoate, diethyl succinate, isoamyl acetate, isobutyl acetate, n-butyl acetate and hexyl acetate);
- 11 alcohols (ethanol, 1-pentanol, 1-hexanol, 1-heptanol, 1-octanol, 1-decanol, 2-hexanol, 2-octanol, 2-methyl-1-butanol, n-butanol and 3-methyl-1-butanol);
- 5 ketones (2,3-butanedione, 2- pentanone, 2-hexanone, 2-octanone and 2-heptanone);
- 3 aldehydes (acetaldehyde, benzaldehyde and 3-methyl-butanal);
- 2 organic acids (acetic and caprylic acids).

3.6. Coffee cup quality evaluation

Green coffee samples were roasted in a semi-industrial roaster (Probatino, Leogap model, Brazil) with capacity of 1300 g through the sensory markers technique (PEREIRA et al., 2016). Cup quality was assessed by a panel of four expert coffee tasters with Q-Grader Coffee Certificate, coordinated by Ensei Neto, titular member of the Technical Standards Committee SCAA (2004–2010), according with the procedures described in Pereira et al. (2016).

3.7. Co-inoculation of Lactobacillus plantarum LPBR01 and Lactobacillus paracasei spp. paracasei LPBL07

To evaluate the metabolic behavior in co-inoculation of LAB in coffee processing, *Lactobacillus plantarum* LPBR01 and *Lactobacillus paracasei spp. paracasei* LPBL07 were inoculated under on-farm conditions according to the procedures described above. The differentiation of these two lactic acid bacteria strains were performed according to RICCIARDI et al. (2015).

3.8. Statistical analyses

The data were analyzed in a completely randomized design with three replicates. A Duncan's test was performed using SAS program (Statistical Analysis System-Cary, NC, USA). Level of significance was established in a two-sided p-value < 0.05.

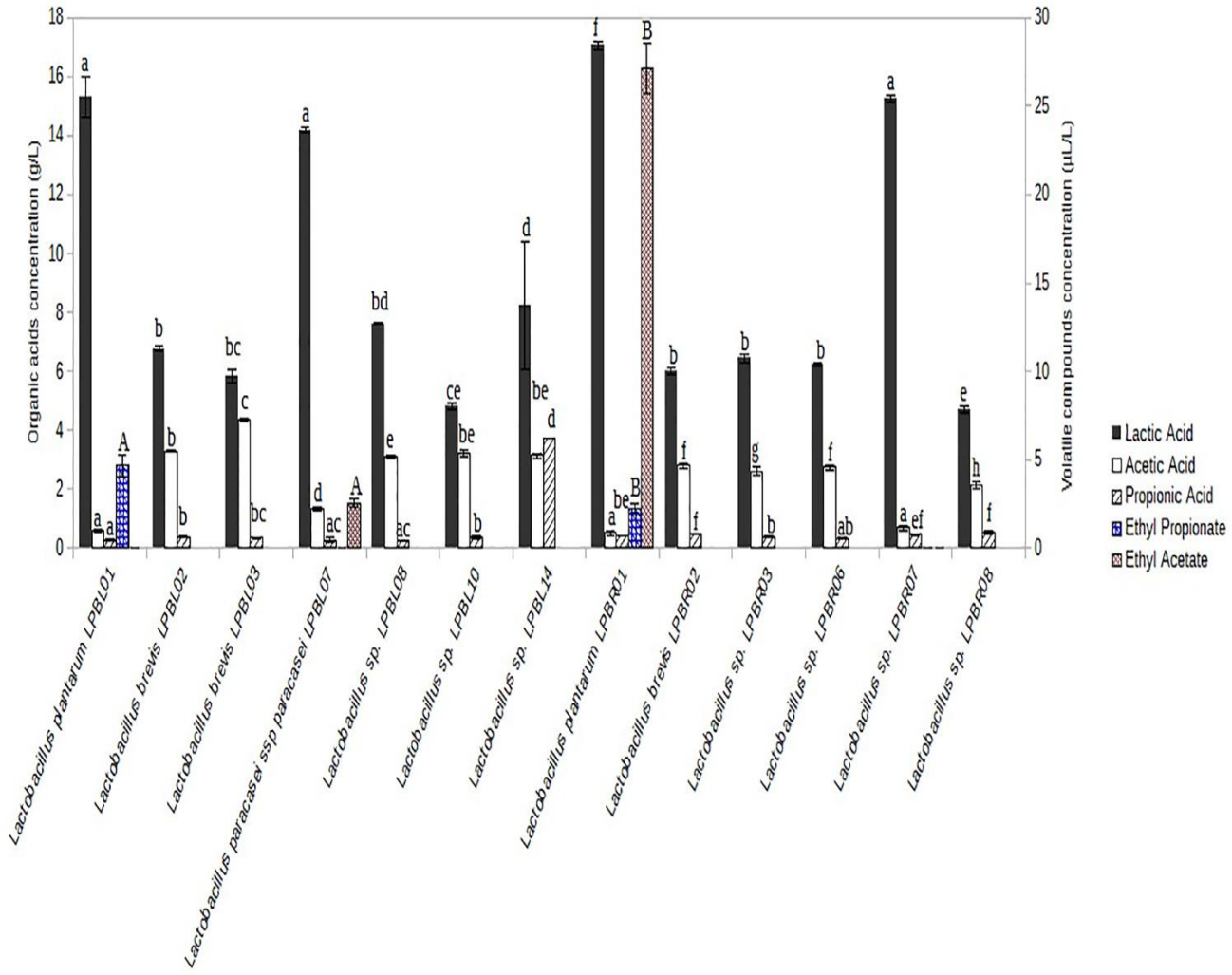
4. RESULTS AND DISCUSSION

4.1. Lactic acid bacteria selection

The organic acids produced during natural coffee bean processing can influence the fermentation performance and beverage quality in different ways. Acids produced of overfermentation, such as butyric, acetic and propionic acids, can damage the final quality of the beverage by conferring an oniony flavor when present at concentrations higher than 1 $mg.mL^{-1}$ (LOPEZ et al., 1989; SILVA et al., 2013). By contrast, lactic acid production can assist in the coffee-pulp acidification process without interfere in the product final quality (PEREIRA et al., 2015b).

In this sense, high production of lactic acid and low of butyric, acetic and propionic acids in a coffee pulp simulation medium was used as primary selection factor to determine the best LAB strain suitable to conduct coffee fermentation process. Based on this, *Lactobacillus* sp. LPBR07, *L. plantarum* LPBL01, *L. plantarum* LPBR01 and *L. paracasei* LPBL07 were preselected and evaluated for their abilities to produce volatile aromatic compounds (FIGURE 1). In this secondary screening, *L. plantarum* LPBR01 produced significantly higher ($p < 0.05$) concentration of flavor-active esters, *viz.* ethyl acetate and ethyl propionate (FIGURE 1). As these compounds are related to fruity aroma, which participates in aromatic complexity of a coffee beverage, this makes *L. plantarum* LPBR01 an attractive LAB strain to enhance the aromatic value of coffee beans and was chosen to evaluate its behavior under field conditions.

FIGURE 1 - CONCENTRATION OF ORGANIC ACIDS AND VOLATILE COMPOUNDS PRODUCED BY DIFFERENT LAB STRAINS IN COFFEE-PULP SIMULATION MEDIUM. UPPER-CASE LETTERS SHOW SIGNIFICANT DIFFERENCES IN VOLATILE COMPOUNDS PRODUCTION AND LOWER-CASE LETTERS SHOW SIGNIFICANT DIFFERENCES IN ORGANIC ACIDS PRODUCTION BY DUNCAN'S TEST.



4.2. Field experiment

The results obtained in the present study showed that *L. plantarum* LPBR01 was able to establish vigorous lactic acid fermentation resulting in accelerated acidification process of the coffee pulp (TABLE 1).

TABLE 1 - TOTAL CELL COUNT (LOG CFU/ML) AND PH MEASUREMENTS OF FERMENTING PULP-MASS BEANS AND SENSORY DESCRIPTIVE TERMS AND TOTAL SCORE OF THE COFFEE BEVERAGE PRODUCED BY THE INOCULATED AND SPONTANEOUS FERMENTATIONS.

Parameters	Fermentation assay					
	Spontaneous			Inoculated		
	T0	T12	T24	T0	T12	T24
LAB	5.49 ± 0.20[a]	5.94 ± 0.03[b]	6.16 ± 0.11[c]	7.35 ± 0.06[d]	9.44 ± 0.05[e]	10.92 ± 0.05[f]
Yeast	4.62 ± 0.15[a]	4.92 ± 0.03[b]	6.30 ± 0.10[c]	4.74 ± 0.13[a]	4.93 ± 0.08[b]	6.31 ± 0.05[c]
pH	5.77 ± 0.06[a]	5.27 ± 0.12[b]	4.50 ± 0.10[c]	5.77 ± 0.12[a]	4.30 ± 0.10[c]	4.37 ± 0.21[c]
Sensory descriptive terms of the beverage	Banana and Papaya (Total score: 80.33±0,57[a])			Banana, pineapple, orange, milky and velvet-like body (Total score: 88.33±1.15[b])		

*Means of triplicate in each row bearing the same letters are not significantly different (p > 0.05) from one another using Duncan's Test (mean ± standard variation).

This is in agreement with the higher counts of LAB in the starter-added fermentation which indicated that *L. plantarum* LPBR01 is competitive and able to grow under coffee-associated environmental conditions. The reduction in pH levels below 4.5 is a widely used method by coffee producers to determine the end of fermentation of the coffee beans during the wet processing (JACKELS; JACKELS, 2005). Thus, the inoculation of *L. plantarum* LPBR01 showed to be a potential alternative way to reduce the fermentation time to 12 h, as against that of 24 h in case of natural fermentation (TABLE 1). The efficient conversion of sugars pulp (glucose and fructose) into organic acids (lactic and fumaric acids) can explain this faster acidification process in the inoculated treatment (TABLE 2).

TABLE 2. CONCENTRATION OF VOLATILE COMPOUNDS (μMOL.L^{-1}), ORGANIC ACIDS AND REDUCING SUGAR (G.L^{-1}) IN FERMENTING COFFEE BEAN MASS OF INOCULATED AND SPONTANEOUS (CONTROL) FERMENTATION.

continue

Compounds	Fermentation assay			
	Spontaneous		Inoculated	
	T0	T24	T0	T24
GC				
Acetaldehyde	ND[a]	63.67 ± 18.67[b]	ND[a]	106.10 ± 8.79[c]
Ethanol	ND[a]	98.72 ± 50.62[ab]	ND[a]	179.15 ± 120.52[b]
2-hexanone	ND[a]	2.50 ± 0.14[b]	ND[a]	ND[a]
Benzaldehyde	ND[a]	1.22 ± 0.49[b]	ND[a]	1.52 ± 0.60[b]
3-methyl-1-butanol	ND[a]	0.65 ± 0.02[b]	ND[a]	ND[a]
2-octanone	ND[a]	1.57 ± 1.08[a]	ND[a]	1.72 ± 1.55[a]
n-Buthyl acetate	ND[a]	0.64 ± 0.03[b]	ND[a]	0.72 ± 0.07[c]
Ethyl acetate	ND[a]	33.85 ± 5.84[b]	ND[a]	64.98 ± 5.85[c]
Ethyl isobutyrate	ND[a]	111.90 ± 1.61[b]	ND[a]	273.95 ± 47.08[c]

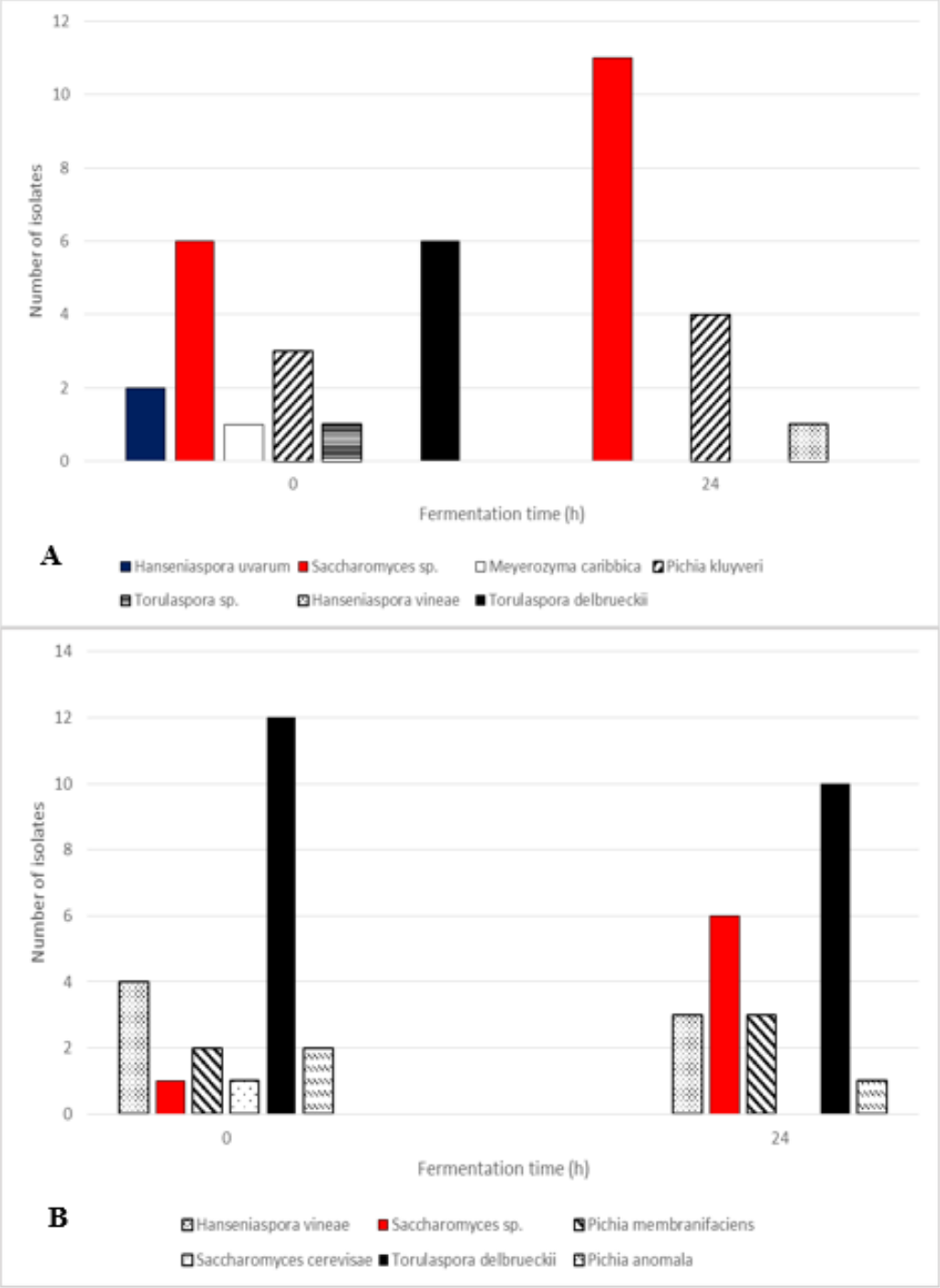
1-octanol	ND[a]	2.30 ± 1.48[b]	ND[a]	5.05 ± 0.84[c]
1-decanol	ND[a]	1.55 ± 0.20[b]	ND[a]	1.57 ± 0.45[b]
Isoamyl acetate	ND[a]	16.90± 7.25[b]	ND[a]	38.33 ± 8.81[c]
Ethyl hexanoate	ND[a]	0.97 ± 0.29[b]	ND[a]	2.07 ± 0.79[c]
3-ethyl-hexanoate	ND[a]	5.13 ± 0.10[b]	ND[a]	1.76 ± 0.29[c]
2-heptanone	15.79 ± 5.28[a]	136.68 ± 14.94[b]	14.46 ± 9.77[a]	121.31 ± 29.78[b]
2-phenylethanol	ND[a]	2.34 ± 0.50[a]	ND[a]	7.16 ± 2.83[b]
Ethyl succinate	ND[a]	1.14 ± 0.20[b]	ND[a]	2.03 ± 0.49[c]
Ethyl octanoate	ND[a]	1.28 ± 0.75[b]	ND[a]	2.16 ± 0.34[c]
2,3-butadione	ND[a]	7.98 ± 0.57[b]	ND[a]	8.10 ± 0.37[b]
Hexyl acetate	ND[a]	ND[a]	ND[a]	6.35 ± 0.80[b]
Ethyl decanoate	ND[a]	ND[a]	ND[a]	1.12 ± 0.56[b]
1-hexanol	1.80 ± 0.77[a]	ND[b]	1.74 ± 1.12[a]	ND[b]
n-propyl alcohol	3.49 ± 0.44[a]	ND[b]	3.40 ± 0.39[a]	ND[b]
Total	21.08 ± 6.36[a]	490.96 ± 65.87[b]	19.603 ± 11.25[a]	824.04 ± 143.28[c]
HPLC				
Glucose	1.75 ± 0.16[a]	0.21 ± 0.11[b]	1.75 ± 0.11[a]	ND[c]
Fructose	1.96 ± 0.19[a]	0.70 ± 0.17[b]	1.99 ± 0.12[a]	ND[c]
Lactic acid	0.05 ± 0.01[a]	0.89 ± 0.07[b]	0.06 ± 0.04[a]	1.33 ± 0.04[c]
Acetic acid	ND[a]	0.27 ± 0.03[b]	ND[a]	0.41 ± 0.01[c]
Citric acid	0.08 ± 0.03[a]	ND[b]	0.08 ± 0.03[a]	ND[b]
Malic acid	ND[a]	ND[a]	ND[a]	0.15 ± 0.01[b]
Succinic acid	ND[a]	0.38 ± 0.16[b]	ND[a]	0.16 ± 0.01[a]
Fumaric acid	0.06 ± 0.01[a]	0.28 ± 0.03[b]	0.06 ± 0.01[a]	0.64 ± 0.02[c]

ND, not detected; GC, gas chromatography; HPLC, high-performance liquid chromatography.
Means of triplicate in each row bearing the same letters are not significantly different ($p > 0.05$) from one another using Duncan's Test (mean standard variation).

The inoculation of *L. plantarum* LPBR01 showed no significant influence on yeast growth (TABLE 1). The results also demonstrated that yeast diversity was not significantly influenced by LAB inoculation (FIGURE 2). The specie of *Torulaspora delbrueckii* and *Saccharomyces* sp. were detected in higher counts in both treatments. These species were also reported previously in studies evaluating diversity in wet and semi-dry processing (MASOUD et al., 2004; VILELA et al., 2010). The presence of yeasts, such as *Saccharomyces*, *Pichia*, *Torulaspora* and *Hanseniaspora*, provides benefits for coffee processing due to pectionolitic enzyme production, production of aromatic compounds and growth inhibition of spoilage or mycotoxigenic fungi (MASOUD et al., 2005; MASOUD; JERPSEN, 2006; SILVA et al., 2008; PEREIRA et

al., 2014; EVANGELISTA et al., 2014; PEREIRA et al., 2014; EVANGELISTA et al., 2015).

FIGURE 2 - DISTRIBUTION AND FREQUENCY OF YEAST SPECIES DURING SPONTANEOUS (A) AND INOCULATED (B) ON-FARM COFFEE WET PROCESSING.



The use of *L. plantarum* LPBR01 also increased significantly ($p < 0.05$) the production of volatile aroma compounds during the fermentation process, such as butyl acetate, ethyl acetate, 1-octanol, isoamyl acetate, ethyl hexanoate, ethyl succinate and ethyl octanoate. Recently, studies have demonstrated that these flavor- active esters can

diffuse into the beans and implement distinct sensory notes to the beverage, despite the kinetics of the event is still not well understood (MASOUD et al., 2005; SILVA et al., 2013; PEREIRA et al., 2015a).

4.3. Coffee cup quality evaluation

Coffee beverages produced from both inoculated and spontaneous processes were sensory evaluated (TABLE 1). The coffee inoculated with *Lactobacillus plantarum* LPBR01 obtained a positive evaluation (88 points SCAA) featuring a velvety-like body, fruity aromas noticeable of "banana", "orange" and "pineapple" and milky flavor. Beverages produced from spontaneous process got a faint aroma of "papaya", being classified as defective (80. points SCAA). Positive attributes such as fruity, floral, acid and caramel notes in the beverages are frequently mentioned and appreciated in coffee cup quality evaluations (LELOUP et al., 2004; NEBESNY; BUDRYN, 2006). The aromas and flavors detected by sensory assessors corroborate the volatile compounds and metabolites detected in quantitative analysis and can be associated with the specific compounds produced by the LAB evaluated.

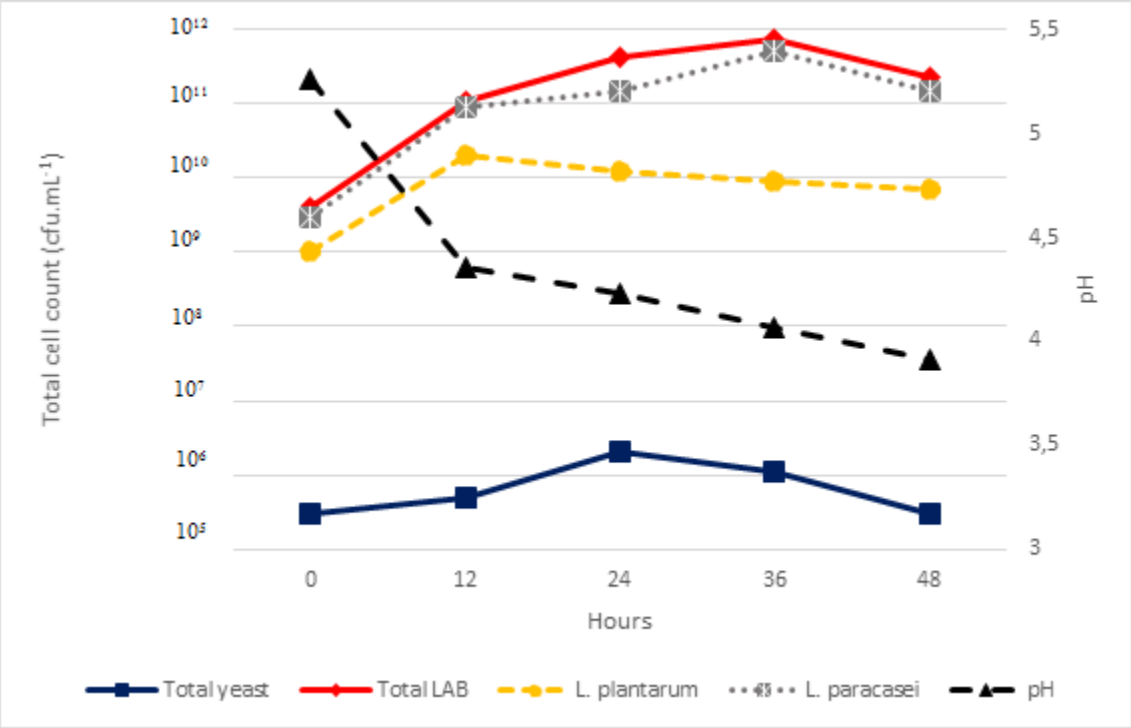
Although no experimental evidence has been given, it is often mentioned that such metabolites might contribute unique fruity, floral, sweet and other notes to food products' character (PEREIRA et al., 2014). Thus, this study demonstrated the increase in the volatile profile and in the sensory characteristics of the beverage due to the LAB starter application. However, more investigations are required with the aim of studying the volatile compounds of the final beverage.

4.4. Co-inoculation of *Lactobacillus plantarum* LPBR01 and *Lactobacillus paracasei* spp. paracasei LPBL07

For this preliminary analysis, the homofermentative *Lactobacillus plantarum* LPBR01 and the heterofermentative *L. paracasei* spp. *paracasei* LPBL07 (previously selected and displayed in FIGURE 1). were co-inoculated under on-farm conditions. Total LAB and yeast counts and pH values are showed in FIGURE 3. It was possible to verify a strong dominance of LAB over yeast population (FIGURE 3). The good adaptation of LAB under coffee-associated environmental conditions may decrease the bioavailability of nutrients for yeast growth.

Although showing a similar growth during the first 12 h of fermentation, the *Lactobacillus plantarum* LPBL07 become dominant and reached a maximum count of 5.2 x 10^{11} cfu.mL^{-1} against 2 x 10^{10} cfu.mL^{-1} of the strain LPBR01. The co-inoculation of both these strains could bring advances for coffee bean fermentation performance. As the strain LPBR01 is a homofermentative LAB, hexoses are catabolized *via* the pyruvate to a restricted lactic acid formation promoting rapid acidification of coffee pulp. On the other hand, LPBL07 strain carries out oxidation followed by decarboxylation of hexoses, generating products such as acetic acid, ethanol and others aromatic compounds (*i.e.,* acetaldehyde and diacethyl) (KANDLER, 1983). This can add complexity in the final chemical composition of coffee beans and should be better studied in futures work.

FIGURE 3 - TOTAL YEAST, LAB, *LACTOBACILLUS PLANTARUM* AND *L. PARACASEI* SPP. *PARACASEI* COUNTS AND PH MONITORING OF THE INOCCULATED COFEE WET PROCESSING.



5. CONCLUSION AND PERSPECTIVE

The results obtained in this study suggest that LAB, previously unexplored as starter culture in coffee processing, play a crucial role in the fermentation performance and beverage quality. The implementation of the selected *L. plantarum* LPBR01 in coffee processing promoted the dominance of LAB without interfering with the yeast growth and diversity and drastically reduced the time of fermentation. Furthermore, the coffee beverage produced by inoculated process showed distinct sensory notes and a remarkable increase in quality compared with the conventional process. The association of heterofermentative (*L. paracasei* spp. *paracasei* LPBL07) and homofermentative (*L. plantarum* LPBR01) strains brings new avenues for adding chemical complexity for coffee bean fermentation process.

Further research are needed to prove the dominance of the inoculated strain. It is also suggested a detailed study of the metabolic interaction between the two LAB, assessing the production of organic acids and volatile compounds, consumption of reducing sugars and their influence on yeast diversity.

ACKNOWLEDGMENTS

I appreciate the Conselho Nacional de Desenvolvimento Científico e Tecnológico do Brasil (CNPq) for the research scholarship and the Universite Aix Marseille (AMU) for the partnership provided in this work.

REFERENCES

ARRUDA N.P., HOVELL A.M.C., REZENDE C.M., FREITAS S.P., COURI S., BIZZO H.R. **Correlation between precursors and volatiles in Brazilian Arabica coffee processed by dry, semi-dry and wet methods and discrimination by principle component analysis.** Química Nova, 35(10):2044-2051, 2012.

AVALLONE S., GUYOT B., BRILLOUET J.M., OLGUIN E., GUIRAUD J.P. **Microbiological and biochemical study of coffee fermentation.** Current Microbiology, 42:252-256, 2001.

AVALLONE S., BRILLOUET J.M., GUYOT B., OLGUIN E., GUIRAUD J.P. **Involvement of pectinolytic micro-organisms in coffee fermentation**. International Journal of Food Science and Technology, 37(2):191-198, 2002.

BATISTA L.R., CHALFOUN S.M., SILVA C.F., CIRILLO M., VARGA E.A., SCHWAN R.F. **Ochratoxin A in coffee beans (*Coffea arabica* L.) processed by dry and wet methods**. Food Control, 20:784-790, 2009.

BUTTY M. **Rapid fermentation of coffee**. Kenya Coffee, 38:214-224, 1973.

CAGLIANI L.R., PELLEGRINO G., GIUGNO G., CONSONNI R. **Quantification of Coffea arabica and Coffea canephora var. robusta in roasted and ground coffee blends.** Talanta, 106:169-173, 2013.

CALLE V.H. **Activadores bioquímicos para la fermentation del café.** Cemicafé, 8:94-101, 1957.

DE BRUYNE K., SCHILLINGER U., CAROLINE L., BOEHRINGER B., CLENKWERCK I., VANCANNEYT M. ***Leuconostoc holzapfelii* sp. nov., isolated from Ethiopian coffee fermentation and assessment of sequence analysis of housekeeping genes for delineation of *Leuconostoc* species.** International Journal of Systematic and Evolutionary Microbiology, 57:2952-2959, 2007.

EVANGELISTA S.R., MIGUEL M.G.C.P., CORDEIRO C.S., SILVA C.F., PINHEIRO A.C.M., SCHWAN R.F. **Inoculation of starter cultures in a semi-dry coffee (*Coffea arabica*) fermentation process.** Food Microbiology, 44:87–95, 2014.

EVANGELISTA S.R., MIGUEL M.G.C.P., SILVA C.F., PINHEIRO A.C.M., SCHWAN R.F. **Microbiological diversity associated with the spontaneous wet method of coffee fermentation.** International Journal of Food Microbiology, 210, 102-112, 2015.

HUCH M., AND FRANZ C.M.A.P. Coffee: fermentation and microbiota. In: **Advances in fermented foods and beverages**, pp. 501-513, 2015.

ICO. **International Coffee Organization. Statistics.** Available in: <http://www.ico.org>. (Access at: March, 2016).

JACKELS S.C., AND JACKELS C.F. **Characterization of the coffee mucilage fermentation process using chemical indicators: A field study in Nicaragua.** Journal of Food Science, 70(5):321-325, 2005.

JOËT T, LAFFARGUE A., DESCROIX F., DOULBEAU S., BERTRAND B., KOCHKO A., DUSSERT S. **Influence of environmental factors, wet processing and their interactions on the biochemical composition of green Arabica coffee beans.** Food Chemistry, 118:693-701, 2010.

KANDLER O. **Carbohydrate metabolism in lactic acid bacteria.** Antonie van Leeuwenhoek, 49, 209-224, 1983.

LEE L.W., CHEONG M.W., CURRAN P., YU B., LIU S.Q. **Coffee fermentation and flavor – An intricate and delicate relationship.** Food Chemistry, 185:182-191, 2015.

LELOUP V., GANCEL C., LIARDON R., RYTZ A., PITHON A. **Impact of wet and dry processing on green coffee composition and sensory characteristics.** Amino Acids, 93-101, 2004.

LEONG K.H., CHEN Y.S., PAN S.F., CHEN J.J., WU H.C., CHANG Y.C., YANAGIDA F. **Diversity of lactic acid bacteria associated with fresh coffee cherries in Taiwan.** Current Microbiology, 68:440-447, 2014.

LOPEZ C.I., BAUTISTA E., MORENO E., DENTAN E. **Factors related to the formation of. ‘‘overfermented coffee beans’’ during the wet processing method and storage of coffee.** ASIC, 13 Colloque, Paipa, 1989.

MASOUD W., CESAR L.B., JESPERSEN L., JAKOBSEN M. **Yeast involved in fermentation of *Coffea arabica* in East Africa determined by genotyping and by direct denaturating gradient gel electrophoresis.** Yeast, 21, 549–556, 2004.

MASOUD W., POLL L., JAKOBSEN M. **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, 2005.

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

NEBESNY E., AND BUDRYN G. **Evaluation of sensory attributes of coffee brews from robusta coffee roasted under different conditions.** European Food Research and Technology, 224, 159-165, 2006.

OLIVEIRA L.S., FRANCA A.S., MENDONÇA J.C.F., BARROS-JÚNIOR M.C. **Proximate composition and fatty acids profile of green and roasted defective coffee beans.** LWT – Food Science and Technology, 39:235-239, 2006.

PEREIRA G.V.M., SOCCOL V.T., PANDEY A., MEDEIROS A.B.P., LARA J.M.R.A., GOLLO A.L., SOCCOL C.R. **Isolation, selection and evaluation of yeasts for use in fermentation of coffee beans by the wet process.** International Journal of Food Microbiology, 188:60-66, 2014.

PEREIRA G.V.M., NETO E., SOCCOL V.T., MEDEIROS A.B.P., WOICIECHOWSKI A.L., SOCCOL C.R. **Conducting starter culture-controlled fermentations of coffee beans during on-farm wet processing: Growth, metabolic analyses and sensorial effects.** Food Research International, 75:348-356, 2015a.

PEREIRA G.V.M., SOCCOL V.T., BRAR S.K., NETO E., SOCCOL C.R. **Microbial ecology and starter culture technology in coffee processing.** Critical Review in Food Science and Nutrition, 1-48, 2015b.

PEREIRA G.V.M., BEUX M., PAGNONCELLI M.G.B., SOCCOL V.T., RODRIGUES C., SOCCOL C.R. **Isolation, selection and evaluation of antagonistic yeasts and lactic acid bacteria against ochratoxigenic fungus *Aspergillus westerdijkiae* on coffee beans.** Letters in Applied Microbiology, 62(1), 96–101, 2016.

RICCIARDI A., PARENTE E., TRAMUTOLA A., GUIDONE A., IANNIELLO R.G., PAVLIDIS D., TSAKALIDOU E., ZOTTA T. **Evaluation of a differential medium for the preliminary identification of members of the *Lactobacillus plantarum* and *Lactobacillus casei* groups.** Annals of Microbiology, 65, 1649-1658, 2015.

SELMAR D., BYTOF G., AND KNOPP S. New aspects of coffee processing: The relation between seed germination and coffee quality. In: **Proceedings of the international congress of ASIC**, Paris: Trieste, pp. 14-18, 2002.

SILVA C.F., SCHWAN R.F., DIAS E.S., WHEALS A.E. **Microbial diversity during maturation and natural processing of coffee cherries of *Coffea arabica* in Brazil.** International Journal of Food Microbiology, 60:251-260, 2000.

SILVA C.F., BATISTA L.R., ABREU L.M., DIAS E.S., SCHWAN R.F. **Succession of bacterial and fungal communities during natural coffee (*Coffea arabica*) fermentation.** Food Microbiology, 25:951-957, 2008.

SILVA C.F., VILELA D.M., DE SOUZA C.C., DUARTE W.F., DIAS D.R., SCHWAN R.F. **Evaluation of a potential starter culture for enhance quality of coffee fermentation.** World Journal of Microbiology and Biotechnology, 29:235–247, 2013.

STEINKRAUS K.H. **Industrialization of indigenous fermented foods.** CRC Press, Boca Raton, 2004.

SUNARHARUM W.B., WILLIAMS D.J. AND SMYTH H.E. **Complexity of coffee flavor: A compositional and sensorial perspective.** Food Research International, 62:315-325, 2014.

TAMIME A.Y. Microbiology of starter cultures. In: R. K. Robinson (Ed.), **Dairy Microbiology handbook**, New York: Wiley-Interscience, pp. 261-366, 2003.

VELMOUROUGANE K., BHAT R., GOPINANDHAN T.N., PANNEERSELVAM P. **Management of *Aspergillus ochraceus* and Ochratoxin-A contamination in coffee during on-farm processing through commercial yeast inoculation**. Biological Control, 57:215-221, 2011.

VILELA D.M., PEREIRA G.V.M., SILVA C.F., BATISTA L.R., SCHWAN R.F. **Molecular ecology and polyphasic characterization of the microbiota associated with semi-dry processed coffee (*Coffea arabica* L.).** Food Microbiology, 27:1128–1135, 2010.

ANEXES

TABLE 3. Coffee-pulp simulation medium constitution.

Composition	Concentration ($g.L^{-1}$)
Citric pectin [Sigma]	2.0
Fructose	15.0
Glucose	15.0
Yeast extract	5.0
Soya peptone	5.0
Coffee-pulp extract	-

The coffee-pulp extract was prepared using 200 g of coffee pulp and peel mixed with 1 L of sterile water with the aid of a blender during 5 minutes.

FIGURE 4 - REPETITIVE SEQUENCE BASED POLYMERASE CHAIN REACTION (Rep-PCR) FINGERPRINTS OF YEAST ISOLATES.