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Cocoa fermentation: Microbial identification by MALDI-TOF MS, and sensory evaluation of produced chocolate

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A B S T R A C T

Dynamic microbial over the cocoa fermentation using starter culture and the effect sensory characteristics of chocolate produced were investigated. The cocoa fermentation inoculated with *Saccharomyces cerevisiae* CCMA0681 and *Lactobacillus fermentum* UFLA CHBB 8.12 as starter cultures were assessed, and compared with spontaneous fermentation. The microbial succession was identified using polyphasic approach including classical morphological and biochemical assays, and Matrix Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF). Overall 873 colonies were isolated, 445 (51%) strains were isolated from the spontaneous fermentation, while 428 (49%) strains were isolated from the inoculated fermentation. The dominant yeast in both fermentation processes were *S. cerevisiae* and *Candida magnolia*. *L. fermentum* and *Pediococcus acidilactici* were detected in both fermentations, whereas *L. coryniformis*, *L. curvatus*, *L. mali*, *L. plantarum*, and *L. sakei* were isolated from the spontaneous fermentation only. *Acinetobacter* sp., *Gluconobacter oxydans*, and *Acetobacter pasteurianus* were isolated from both the fermentation processes. Chocolate produced from the spontaneous fermentative process presented dominance of the bitter flavour, while obtained through inoculated fermentation process presented bitter, astringent and acid as dominant flavours. Cocoa inoculation with *S. cerevisiae* and *L. fermentum* affected the sensory quality of the produced chocolate. The microbial inoculation influenced on fermentation and therefore the final product.

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1. Introduction

Brazil was in the middle of 1980's was the second largest world producer of cocoa, producing approximately 400,000 tons (FAO, 2014). However, at the end of decade, the fungus *Moniliophthora pernicious* (which causes witches' broom disease) brought about a crisis in the Brazilian cocoa industry. From 1990 to 2012, the average yearly production plummeted to 243,386.20 tons of cocoa beans, a drop of 34.25% (FAO, 2014). As result of the introduction of this plant pathogen in Brazil, the search for resistant and more productive cocoa varieties has intensified (Efraim et al., 2010).

Microbial succession during cocoa bean fermentation has already been evaluated previously (Schwan, Pereira, & Fleet, 2014; Schwan, Rose, & Board, 1995). In addition, microbial succession during fermentation of different cocoa hybrids, as well as its effect on the quality of the final product, has been accurately studies previously (Moreira, Miguel, Duarte, Dias, & Schwan, 2013; Nielsen et al., 2007; Schwan, 1998).

Microorganisms play an important role in the food industry, for example as starter cultures for cheese, yogurt, or other fermented foods (Lagunes-Galvez, Loiseau, Paredes, Barel, & Guiraud, 2007; Pereira, Miguel, Ramos, & Schwan, 2012; Pereira, Magalhães, Almeida, Coelho, & Schwan, 2013). Further, to achieve a better understanding of which microorganisms are involved in the

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fermentation process of cocoa, cheese, corn, sorghum, etc., a polyphasic taxonomy of food-related microorganisms is required (Moreira et al., 2013; Pereira et al., 2013; Santos, Ventura, Costa, Fernandes, & Lima, 2015).

Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) emerged in the late 1980s. It is performed to investigate molecular masses of organic compounds, through a soft ionisation of molecules that causes minimum fragmentation. MALDI-TOF MS allows microbial identification at the species and strain levels. It has yielded important scientific contributions as it is an effective tool for rapid identification of microorganisms from different sources (Nicolau, Sequeira, Santos, & Mota, 2014; Oliveira et al., 2015; Passarini, Santos, Lima, Berlinck, & Sette, 2013; Santos et al., 2015).

In this study, the microbial population present during the fermentation of cocoa beans was identified, using a polyphasic approach including MALDI-TOF MS, and morphological and biochemical analyses. Moreover, the effect of *Saccharomyces cerevisiae* CCMA0681 and *Lactobacillus fermentum* UFLA CHBB 8.12 starter cultures on the produced chocolate was assessed. A sensory method based on Temporal Dominance of Sensations (TDS) analysis was applied to compare the inoculated and spontaneous fermentation processes.

2. Materials and methods

2.1. Fermentation and sampling

Ripe cocoa beans PH16 hybrids were fermented at the Vale do Juliana farm in Igrapiúna, Bahia, Brazil. Approximately 100 kg of cocoa was fermented into wooden boxes. Two experiments were performed in parallel, as previously described (Moreira et al., 2013): one spontaneous (non-inoculated) fermentation and one that was inoculated with the starter cultures *S. cerevisiae* CCMA0681 (10^5 cells ml^{-1}) and *L. fermentum* UFLA CHBB 8.12 (10^4 cells ml^{-1}). The cocoa pods were manually opened with a machete, and the beans were immediately transferred to the fermentation house. The fermentation started approximately 4 h after the breaking open the pods and was performed in 0.06 m^3 wooden boxes. Each fermentation was conducted in triplicate. Both strains of starter cultures were inoculated at the beginning of the fermentation process. The initial pH of both fermentations was 4.5, and at the end was 3.5 and 2.5 in the spontaneous and inoculated fermentation, respectively. The initial temperature was 22.2 °C and 44.8 °C at the end of spontaneous fermentation. In the inoculated fermentative process, the initial temperature was 24.5 °C and 46.8 °C at the end of the fermentation (156 h).

For microbiological analyses, samples were taken every 24 h up to 156 h of fermentation. All samples were transferred to the Microbial Fermentation Laboratory (Department of Biology, Federal University of Lavras) for analysis.

2.2. Microbial population dynamics

2.2.1. Microbial count

Yeasts, lactic acid bacteria (LAB), acetic acid bacteria (AAB) and total mesophilic bacteria (MB) cells counts were performed on Yeast Extract Peptone Glucose [YEPG: 1% yeast extract, 2% peptone (Himedia, Mumbai, India), 2% glucose at pH 3.5, containing 100 mgL^{-1} chloramphenicol (Sigma, St. Louis, Missouri, EUA) and 2% agar (Merck, Darmstadt, Germany)] for yeast, De Man Rogosa Sharpe agar [MRS (Merck), containing 0.4% (v/v) nystatin (Merck)] for LAB counting, Glucose Yeast Extract [GYC:5% glucose (Merck), 1 g yeast extract (Merck),3 g calcium carbonate (Merck) and 2 g agar (Merck) per liter, pH 5.6] for AAB and nutrient agar [NA (Merck)] media for total mesophilic bacteria counting.

Twenty-five grams of cocoa beans were transferred into plastic bags containing 225 ml of 0.1% peptone water. The mixture was homogenised for 10 min in Stomacher® (Mayo Homogenius HG 400, São Paulo, Brazil). Sequential 10-fold dilutions were prepared up to a dilution 10^6, using 0.1% sterile peptone water. Yeast cells were enumerated by surface inoculation (0.1 mL^{-1}) on YEPG and plates, which were incubated at 28 °C for 96 h. LAB cells were enumerated by pour-plate inoculation in MRS, and plates were subsequently incubated at 35 °C for 96 h. ABB and MB cells were enumerated by surface inoculation on GYC and NA, and plates were incubated at 28 °C for 96 h.

The number of colony forming units ($CFUml^{-1}$) was assessed at the end of the incubation period. For subsequent tests, the number of representative isolates for each morphotype was calculated, taking into consideration the square root of each plate morphotype (Senguna, Nielsen, Karapinar, & Jakobsen, 2009). The colony size, type, edge structure, color, texture, appearance, rise, shine, and shape were all assessed. All isolates were purified and stored in specific medium containing 20% glycerol, and stored at −80 °C.

2.2.2. Morphological and biochemical characterisation

Yeasts and bacteria strains were isolated and subjected to macroscopic and micro-morphological analyses and biochemical assays. Yeast strains were characterised by spore formation and by fermentation on different carbon sources (glucose, fructose and sucrose), as previously described by Lima-Neto et al. (2014).

All bacterial strains were characterised morphologically and biochemically via Gram staining, catalase reaction, motility test, sporulation and oxidase activity (Holt, Krieg, Sneath, Staley, & Williams, 1994, p. 787). Bacteria cells were grown on MRS medium and underwent fermentation tests using different carbon sources: glucose, maltose, mannitol and sorbitol. Furthermore, the bacterial cells were grown on GYC medium. Strains that were gram-negative and oxidase-negative, were assessed using the Bactray I and II kits for cluster analysis. For gram-negative but oxidase-positive strains, the Bactray III kit was used.

2.2.3. MALDI-TOF sample preparation, measurement and data analysis

Based on the results from the morphological and biochemical characterization, strains were selected for MALDI-TOF MS analysis. Cells were grown on plates using specific culture medium for each taxonomic group, as described above. Cells were incubated at 28 °C for 18 h and then approximately 3×10^7 cells of each strain were aseptically transferred to microtubes. Subsequently, 3 μL of organic solution (water/acetonitrile/trifluoro-acetic acid, 50:47.5:2.5) were added to each microtube containing the bacterial isolates, and 3 μL of formic acid/acetonitrile (25:75) was added to yeast isolates.

The microtubes were immediately and vigorously vortexed for 1 min and then 1 μL of resulting suspension was transferred to the 96-well MALDI flex target plate (Bruker Daltonics, Bremen, Germany). When the liquid phase was almost evaporated 1 μL matrix solution [saturated solution of α-cyano-4-hydroxy-cinnamic acid (CHCA) in 50% acetonitrile/2.5% trifluoro-acetic acid] was added and the solution was gently mixed (Oliveira et al., 2015).

An *Escherichia coli* K12 colony was obtained from the Public Portuguese Culture Collection Micoteca da Universidade do Minho (MUM, www.micoteca.deb.uminho.pt) and used for *in situ* extraction of proteins, which were used as standard for the MALDI-TOF MS external calibration. Cells of *E. coli* K12 were grown on Luria-Bertani medium agar (LB −1% bacto-tryptone, 0.5% bactoyeast extract, 1% NaCl per liter) at 37 °C for 18 h. Briefly, approximately 1 μg of cellular material from a single *E. coli* K12 colony was transferred from the plate to the MALDI flex plate and CHCA matrix

solution was added followed by gently mixing. Each MALDI-TOF sample was spotted in triplicate to evaluate reproducibility. Samples were then analysed in a MALDI-TOF microflex LT spectrometer (Bruker Daltonics, Bremen, Germany), using the MALDI Biotyper 3.0 automatic system.

For MALDI-TOF microbial identification, the following reference strains the Culture Collection of Agricultural Microbiology (CCMA-UFLA, https://sites.google.com/site/ccmaufla/) were used: *Candida orthopsilosis* (CCMA 0571), *Hanseniaspora uvarum* (CCMA 0580), *H. opuntiae* (CCMA 0584), *Pichia kluyveri* (CCMA 0574), *Pichia guilliermondii* (CCMA 0569), *Rhodotorula mucilaginosa* (CCMA 0568), *P. carribica* (CCMA 0559), *S. cerevisiae* (CCMA0681), *Lactobacillus plantarum* (CCMA 0658), *L. fermentum* (UFLA CHBB 8.12), *Leuconostoc pseudomesenteroides* (CCMA 0652), *Acetobacter pasteurianus* (CCMA 0634), *A. aceti* (CCMA 0636) and *Gluconobacter oxydans* (CCMA 0625).

2.3. Sensory analysis

2.3.1. Chocolate preparation

In order to obtain liquor of each fermentation process, fermented and dried beans were processed by Sartori and Pedroso Alimentos Ltda. (São Roque, São Paulo, Brazil). Chocolates with 70% cocoa and 30% icing sugar content were produced by a conventional method, according to Efraim et al., 2010. The chocolates molded (20 g) were wrapped and stored at 18 °C for sensory analysis.

2.3.2. Selection and training of assessors

The sensory evaluation was initially performed with a group of 50 consumers, using the sequential method of Wald's sequential analysis for subject selection (Amerine, Pangborn, & Roessler, 1965, p. 802). In this test, were used two samples of dark chocolate. To determine the Wald's sequential analysis, the following values were used: $P = 0.30$ (maximum incapability acceptable), $P1 = 0.70$ (minimum acceptable skill) and the risks $\alpha = 0.10$ (probability of accepting a candidate without sensory acuity) and $\beta = 0.10$ (probability of rejecting an applicant with sensory acuity). Wald chart was built and the tasters were selected or rejected according to the number of correct tests on a total number of tests performed (Morais, Pinheiro, Nunes, & Bolini, 2014). Afterwards, 20 tasters between 18 and 25 years old were selected. The total analysis time was 40 s with a delay of 3s (start of chewing). Outcomes were analyzed using the statistical program SensoMaker was performed (Nunes & Pinheiro, 2012).

2.3.3. Temporal Dominance of Sensations (TDS)

The attributes involved in the analysis of TDS were established by the traditional method (Stone, Bleibaum, & Thomas, 2012, p. 438). The consensual descriptive terms were summarized in a discussion group. The eight most relevant attributes in relation to the flavours perceived in chocolates were defined. The selected attributes were: bitter, sour, sweet, cocoa flavour, wood/tobacco, astringent, fruit and no flavour. Through the pre-tests, tasters were asked to describe the attributes related to the flavour present in the chocolate samples.

TDS analysis was then performed in triplicate, according to the methodology proposed by Pineau et al. (2009) with the 10 selected and trained tasters, using the SensoMaker program for the collection and analysis of data (Nunes & Pinheiro, 2012). The tasters were asked to choose the dominant flavour over time, described as the taste that is perceived with greater clarity and intensity.

In order to calculate the TDS curves in the SensoMaker software, the methodology of Pineau et al. (2009) was followed. Two lines were drawn on graphics: the “chance level” and the “significance level”. The “chance level” is the dominance level that can be assigned to any attribute by chance. The “significance level” is the minimum value of this ratio to be considered significant.

2.4. Statistical analysis

Each population count was done in triplicate and the mean values ± standard deviations are reported. The sensorial data were recorded using the SENSOMAKER Software (Nunes & 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. Results

3.1. Quantification and characterisation of microbial population

The yeasts, LAB, AAB, and mesophilic aerobic bacteria populations found during the spontaneous fermentation of cocoa beans are shown in Table 1. The initial yeast count in PH16 cocoa beans in the absence of starter culture was 3.3 log CFUml^{-1}. This population remained constant throughout the spontaneous fermentation process during the first 60 h. After that, the count decreased to a final value of 1.52 log CFUml^{-1} at 156 h. For inoculated cocoa bean fermentation, the initial yeast count was 4.9 log CFUml^{-1}. This population count was on average 3.66 log CFUml^{-1} up to 108 h of fermentation, but decreased to 1.52 log CFUml^{-1} at 132 h. At the end of the fermentation, yeast levels were below the detection limit (Table 1).

The initial microbial count of LAB in the spontaneous fermentation of cocoa beans was 1.09 log CFU ml^{-1}; which increased to 1.39 log CFUml^{-1} at the end of the fermentation process. In contrast, the initial population of LAB during the inoculated cocoa bean fermentation was 3.74 log CFUml^{-1}. At the end of the

Table 1
Total cell count (log CFUmL^{-1}) of different microorganisms during fermentation of cocoa bean.

Fermentation time (h)								
Spontaneous	0	12	36	60	84	108	132	156
MB	2.99 ± 0.02	3.09 ± 0.10	3.35 ± 0.05	2.17 ± 0.02	3.29 ± 0.02	3.23 ± 0.01	3.55 ± 0.00	2.87 ± 0.01
LAB	1.09 ± 0.01	2.02 ± 0.05	2.69 ± 0.00	2.43 ± 0.10	2.02 ± 0.02	1.95 ± 0.02	1.74 ± 0.00	1.39 ± 0.02
Yeast	3.31 ± 0.00	3.5 ± 0.11	3.34 ± 0.15	3.3 ± 0.00	2.22 ± 0.02	1.82 ± 0.01	1.52 ± 0.00	1.52 ± 0.00
AAB	1.5 ± 0.01	3.61 ± 0.13	2.69 ± 0.02	2.65 ± 0.02	2.69 ± 0.00	2.17 ± 0.07	2.84 ± 0.09	3.3 ± 0.10
Inoculated								
MB	2.97 ± 0.02	2.47 ± 0.01	3.04 ± 0.00	3.04 ± 0.00	2.74 ± 0.10	3.11 ± 0.04	5.4 ± 0.05	3.13 ± 0.00
LAB	3.74 ± 0.13	2.64 ± 0.02	2.29 ± 0.04	2.27 ± 0.02	2.07 ± 0.05	1.69 ± 0.02	1.39 ± 0.02	nd
Yeast	4.95 ± 0.20	3.63 ± 0.02	3.4 ± 0.08	3.95 ± 0.05	3.65 ± 0.09	3.71 ± 0.02	1.52 ± 0.09	nd
AAB	nd	1.17 ± 0.00	2.3 ± 0.02	2.39 ± 0.01	3.91 ± 0.10	3.07 ± 0.05	2.3 ± 0.07	2.69 ± 0.08

Nd = Not detected. Mean ± standard deviation.

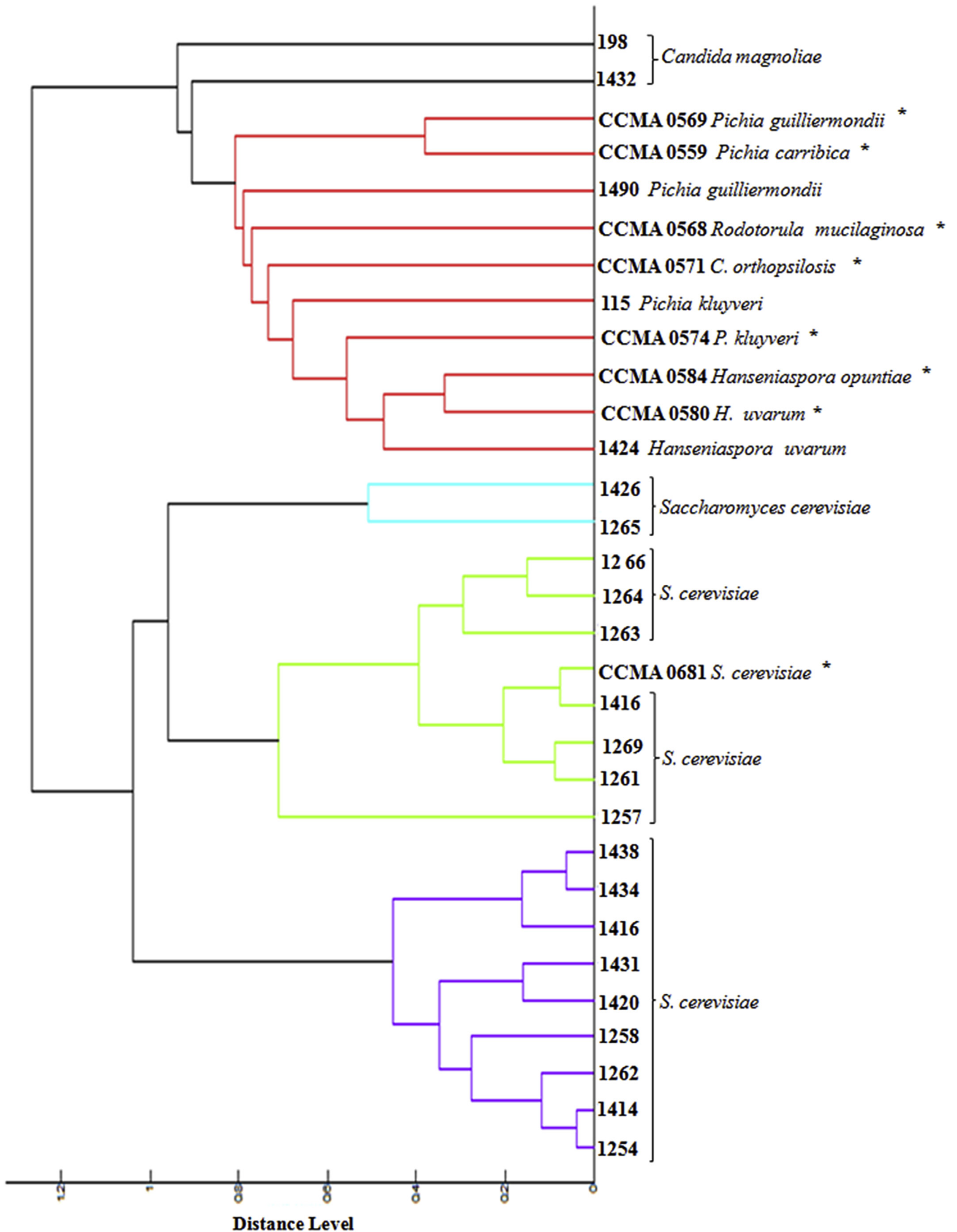

Fig. 1. Cluster analysis of the MALDI-TOF MS reference database spectra of yeasts strains. Distances are measured as a percentage of spectral similarity. *Reference strains.

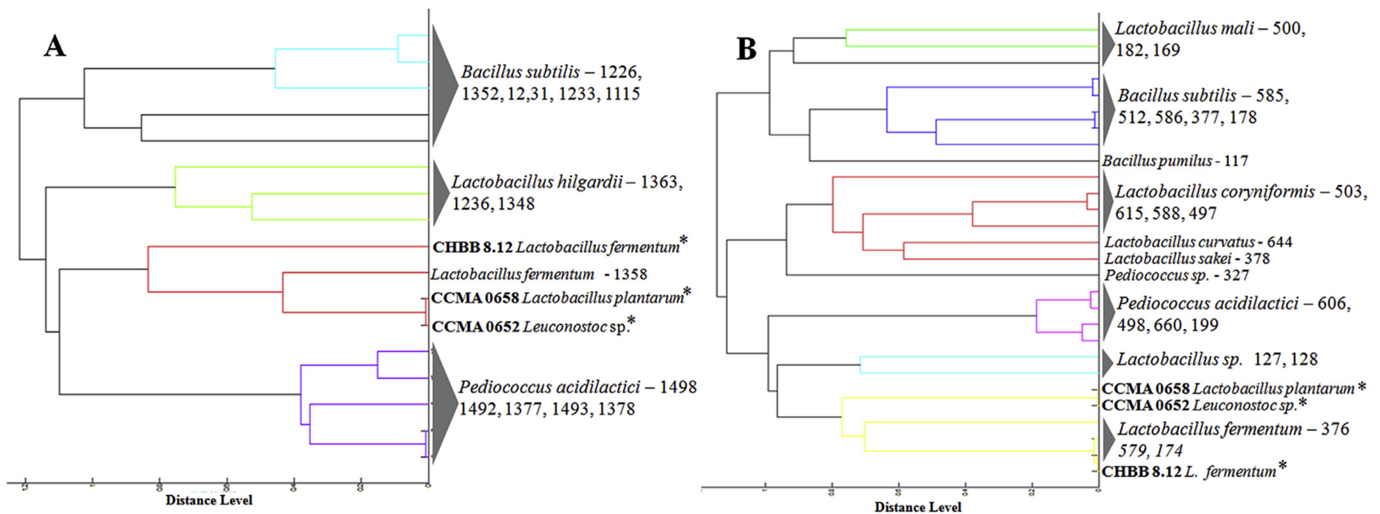

Fig. 2. Dendrogram based on relatedness of proteomic profiles by MALDI-TOF MS for bacteria strains obtained MRS plates. A) non-inoculated cocoa fermentation and B) inoculated cocoa fermentation. Distances are measured as a percentage of spectral similarity; *Reference strains.

fermentation, the levels of this group of microorganisms were negligible (Table 1).

The initial count of AAB during the spontaneous fermentation was 1.51 log CFUml^{-1}. After 156 h of fermentation, the average population count was 3.3 log CFUml^{-1} (Table 1). AAB were not detected at the start of the inoculated cocoa bean fermentation. After 84 h of fermentation, the AAB reached a population count of 3.91 log CFUml^{-1}. However, at the end of the fermentation, the population count decreased to 2.69 log CFUml^{-1} (Table 1).

The initial MB population during the spontaneous fermentation was 2.99 log CFUml^{-1}. The population count decreased to 2.87 log CFUml^{-1} at the end of the process. For this group of microorganisms, the inoculated cocoa bean fermentation showed a slightly lower initial population count of 2.97 log CFUml^{-1}, but at the end of the fermentation (157 h), its levels rose to 3.13 log CFUml^{-1} (Table 1).

Overall, 873 colonies were isolated from both spontaneous and inoculated cocoa bean fermentations. More specifically, 445 (51%) strains were isolated from the spontaneous fermentation (24 yeasts, 139 LAB, 64 AAB and 218 MB), while 428 (49%) strains were isolated from the inoculated fermentation (57 yeasts, 94 LAB, 70 AAB and 207 MB). These isolates were evaluated by different biochemical assays and based on their micro-morphological traits (data not shown), and subsequently identified by MALDI-TOF (Figs. 1–4).

According to the MALDI-TOF MS results, 44 isolates were identified with a BioTyperlog (score) > 1.70 (Fig. 1). The dominant species in both fermentation processes were *S. cerevisiae* and *Candida magnoliae*. The species *Pichia kluyveri* was found in the spontaneous fermentation process only. In contrast, *Hanseniaspora uvarum* and *P. guilliermondii* species were detected in the inoculated fermentation process only.

LAB species identified in both fermentations are shown in Fig. 2. *L. fermentum* and *Pediococcus acidilactici* were detected in both fermentations, whereas *L. coryniformis*, *L. curvatus*, *L. mali*, *L. plantarum* and *L. sakei* were isolated from the spontaneous fermentation only, and *L. hilgardii* was isolated from the inoculated process only.

Acinetobacter sp. *Gluconobacter oxydans* and *Acetobacter pasteurianus* were isolated from both the fermentation processes (Fig. 3).

Bacillus megaterium, B. pumilus, *B. pseudomycoides*, *B. subtilis*, *Lysinibacillus fusiformis*, were detected in both fermentation processes. In contrast, *B. mojavensis*, *B. patagoniensis*, *Lysinibacillus sphaericus*, *Paenibacillus lautus*, *P. illinoisensis* and *P. chibensis* were found in the spontaneous fermentation process only, and *B. amyloliquefaciens*, *B. altitudinis*, *B. cereus*, *B. jeotgali* and *B. pycnus* were isolated exclusively in the inoculated fermentation. In general, mesophilic bacteria had the highest population counts and greater species diversity in the spontaneous fermentations (Table 1 and Fig. 4).

3.2. Temporal dominance analysis of sensations of chocolate

The TDS profile of the two chocolate samples under study is shown in Fig. 5. The only dominant flavour of the chocolate obtained from the spontaneous fermentation was sour (Fig. 5A). However, other attributes were also highly scored, such as sweetness, acidity, astringency and cocoa flavour. In contrast, the chocolate obtained from the inoculated fermentation process was predominantly perceived as bitter. In addition, acidity and astringency were also frequently perceived (Fig. 5B).

4. Discussion

The largest yeast population was observed during inoculated fermentation of the cocoa beans, probably due to the inoculation with *S. cerevisiae* CCMA 0681 in a population of 10^5 cells mL^{-1}. The dominant yeast species during both fermentation processes were *S. cerevisiae* and *C. magnolia*. This data corroborates that in previous publications (Nielsen et al., 2007; Pereira et al., 2012), which describe the predominance of these two yeast species during fermentation of different cocoa hybrids.

The results from the MALDI-TOF MS spectral clustering for yeast identification are presented are presented in Fig. 1. Two clusters of *Saccharomyces* and non-*Sccharomyces* species were identified. Moreover, in both fermentations, the largest BAL counts were observed after 36 h of fermentation (Table 1). In contrast, previous studies reported higher levels of LAB between 48 h and 96 h of cocoa bean fermentation (Ardhana & Fleet, 2003; Nielsen et al., 2007; Schwan, 1998).

The population count of *Lactobacillus* species remained stable during fermentation upon inoculation with the starter culture *Lactobacillus fermentum* UFLA CHBB 8.12 (Table 1). Our results confirmed those of studies conducted by Ho, Zhao, and Fleet (2015), which suggest that LAB are most likely not essential for successful

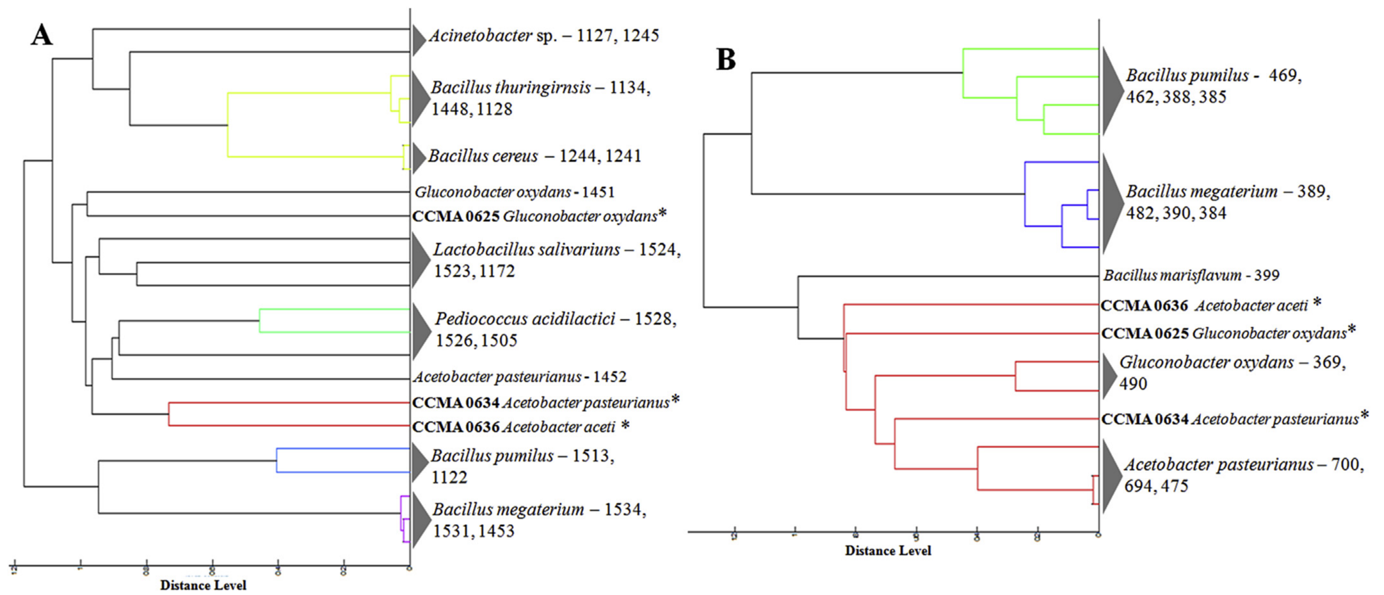

Fig. 3. Dendrogram based on relatedness of proteomic profiles by MALDI-TOF MS for bacteria strains obtained GYC plates. A) non-inoculated cocoa fermentation and B) inoculated cocoa fermentation. Distances are measured as a percentage of spectral similarity; *Reference strains.

cocoa bean fermentation. Dircks (2009), reported that increasing the concentration of ethanol during fermentation of cocoa beans inhibited the growth of *L. fermentum*. This could explain the small population of this group of microorganisms during the first 24 h of fermentation.

The bacterial communities isolated during the both spontaneous and inoculated cacao bean fermentations, and identified by proteomic profiling by MALDI-TOF MS are shown in Figs. 2–4, respectively. The MALDI-TOF MS is an effective method for the identification and detection of different microbial groups. Several

Fig. 4. Dendrogram based on relatedness of proteomic profiles by MALDI-TOF MS for bacteria strains obtained NA plates. A) non-inoculated cocoa fermentation and B) inoculated cocoa fermentation. Distances are measured as a percentage of spectral similarity; *Reference strains.

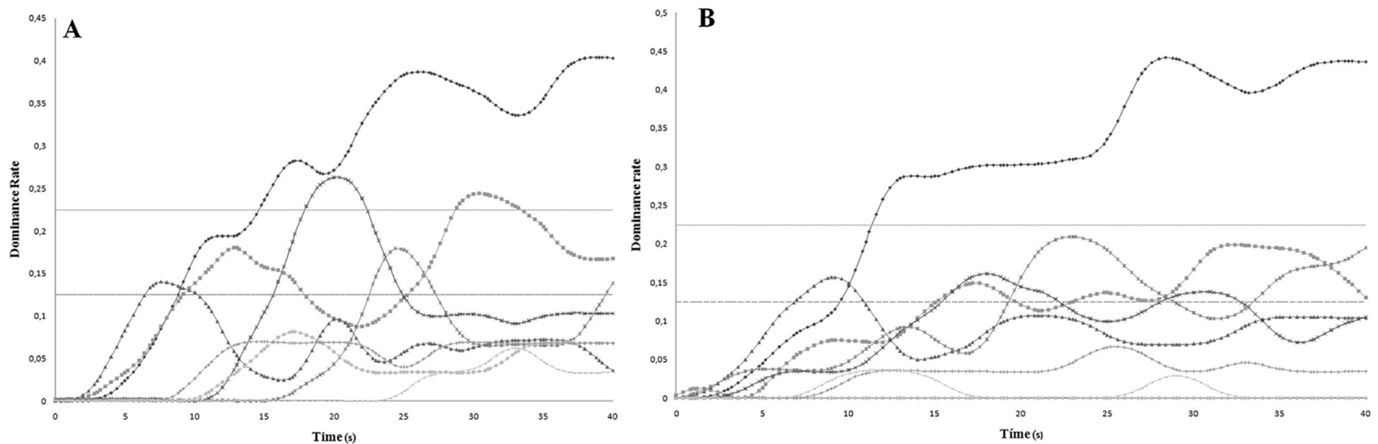

Fig. 5. TDS curves of cocoa fermentation PH 16 hybrid without inoculation (A) and with inoculation (B). Attributed sensations (—•—): Bitter (—■—), sour (—▲—), sweet (—×—), astringent (—•—), cocoa flavor (—•—), fruit (—+—), tobacco (——), tasteless. Significance level (——) and chance level (- - -) duration of 40 s.

studies in different scientific fields have applied this technique for fast microbial identification (Dušková et al., 2012; Pavlovic et al., 2012; Santos et al., 2015).

The spontaneous cocoa bean fermentation process showed a greater variety of species compared to the inoculated fermentations (Figs. 2A, 3A and 4A). This suggests that the starter cultures affected the natural microbial diversity during cocoa fermentation. *L. fermentum* has been used for many years in food preservation, as starter cultures for dairy and meat products, and in fermentation of vegetables (Giraffa, Chanishvili, & Widyastuli, 2010). Further, strains of *L. fermentum* can inhibit the growth of other microbial species (Ramos et al., 2010).

The same AAB species, *Gluconobacter oxydans* and *A. pasteurianus*, were isolated and identified from both fermentation processes. These species have been described in cocoa bean fermentation in Brazil, Ghana and Indonesia (Ardhana & Fleet, 2003; Nielsen et al., 2007; Pereira et al., 2012). Although Nielsen, Arneborg, and Jesspersen (2014), chap. 1 reported that GYC is a suitable medium for the isolation of AAB strains, our MALDI-TOF MS results showed that the main isolated bacterial species in this type of GYC medium were MB and LAB.

In both fermentation processes, several bacterial species were isolated on the NA medium and further identified by MALDI-TOF MS (Fig. 4). Some of the species isolated in this culture medium were previously described for cocoa bean fermentation, namely: *Bacillus. cereus*, *B. megaterium*, *B. pumilus*, and *B. subtilis* (Ardhana & Fleet, 2003; Nielsen et al., 2007; Pereira et al., 2012). The microbial activity in the cocoa bean fermentation leads to increased temperatures, which in turn affects the microbial populations. Consequently, *Bacillus* spp. Have a competitive advantage to survive and grow during the later stages of the fermentation process (Nielsen et al., 2014, chap. 1).

Overall, the inoculation of cocoa with *S. cerevisiae* and *L. fermentum* affected the sensory quality of the produced chocolate. In particular, a lower dominance rate of cocoa flavour and sensorial characteristics such as bitterness, astringency and acidity was observed (Fig. 5). The volatilisation rate of some acids (e.g. acetic acid) during the inoculated fermentation process could be less efficient than during the non-inoculated process. Efraim et al. (2010) reported that acid volatilization could directly affect the flavour of the produced chocolate.

The TDS analysis showed that the chocolates produced from the spontaneous fermentation process was predominantly perceived as sour and astringent (Fig. 5A). The sour sensation was also predominant form chocolate from the inoculated fermentation (Fig. 5B). According to Hoskin and Dimick (1994, p. 107), in addition to fermentation, roasting and conching steps are of utmost importance for the production of quality chocolates. Another explanation for the above results is the use of LAB as starter culture. According to Ho, Zhao, & Fleet (2014), chocolates made with this group of microorganisms are more acidic and lack characteristic chocolate sensations, as was observed in this study.

5. Conclusion

Microbial succession of yeast and bacteria species belonging to the genera *Candida*, *Hanseniaspora*, *Pichia*, *Saccharomyces*, *Lactobacillus*, *Pediococcus*, *Acinetobacter*, *Gluconobacter*, *Acetobacter*, *Bacillus*, and *Paenibacillus* was observed. The species were identified by a polyphasic approach including MALDI-TOF MS and morphological and biochemical assays. MALDI-TOF MS was effective at identifying different microbial species in both spontaneous and inoculated fermentation processes.

The use of the starter cultures *S. cerevisiae* CCMA 0681 and *L. fermentum* UFLA CHBB 8.12 affected the sensory quality of the produced chocolate. According to the TDS analysis, the chocolates obtained from the non-inoculated fermentation elicited predominant bitter and astringent sensations. The bitter sensation was also predominant for the chocolate from the inoculated fermentation process. These results can be explained by the lower organic-acid volatilisation rate in the chocolate obtained from the non-inoculated fermentation process.

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