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Impact of *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* starter cultures on cocoa beans fermentation

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Running title: Yeast inoculation in cocoa beans fermentation

Abstract: Aim of this work was to study the impact of mixed cultures of *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* and *T. delbrueckii* monoculture on the fermentation process conducted on two different cocoa hybrids, PS1319 and SJ02, in Bahia, Brazil. This was performed throughout studying physico-chemical changes during the fermentation process and analyzing volatile compounds and sensory analysis of chocolates. $(GTG)_5$-PCR fingerprinting was used to type isolates at strain level allowing to assess the implantation of the starter cultures added. Resulted clusters were composed by *T. delbrueckii* strains isolated during the first 24 hours of fermentation. On the contrary, *S*. *cerevisiae*, the most strongly fermenting ethanol-tolerant species, took over the fermentation at a second stage. Quantification data of *T*. *delbrueckii* during spontaneous fermentation confirm the attitude of this species of not being so commonly involved in this process.

This study also showed that the inoculum influenced the PS1319 hybrid end-product quality, changing analytic profile and sensory perception of chocolates. No big influences were recorded for SJ02 hybrid, but this may be improved. In combination with *S. cerevisiae*, *T. delbrueckii* had a positive influence on the analytical profile of chocolates. The application of starter cultures did change the aroma profile of the resulting chocolate as determined by GC–MS; in some case the differences observed had a significantly impact on the consumer perception of the chocolates.

Key-words: cocoa beans fermentation; starter culture; Rep-PCR; HPLC; volatile compounds

1. Introduction

In Brazil, like in some other part of the world, within the farmers it is common to cultivate different cocoa varieties in the same field in order to prevent destruction of the cocoa trees by fungus. Therefore, beans from different variety are collected and spontaneously fermented in the same boxes. This procedure can affect the quality of the chocolate as recently reported by Ramos et al. (2014), indeed the variability due to cocoa variety is an important factor influencing end-products quality, together with the fermentation process itself (Schwan and Wheals, 2004).

PS1319 and SJ02 are cocoa varieties produced by CEPLAC (Comissão Executiva do Plano da Lavoura Cacaueira, Bahia, Brazil). These varieties show high productivity and *Moniliophthora perniciosa* resistance (Mandarino and Sena Gomes, 2009).

The cocoa fermentation is still traditional and uncontrolled and an adequate process together with an appropriate selection and handling of raw materials, drying and roasting can influence characteristics of chocolate (Ramos et al., 2014). Fermentation process involves complex microbial activities and biochemical changes that have been recently deeply reviewed by Schwan and Fleet (2014). The microorganisms responsible for the fermentation are yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB), moreover species of *Bacillus* sp., other bacteria and filamentous fungi could also grow with consequent influence on quality of the process. Recently, Ho et al. (2014) elucidated the importance of yeast growth and activity for successful cocoa bean fermentation and their involvement on the development of chocolate aroma.

Torulaspora delbrueckii is not normally involved in cocoa beans fermentation, indeed this species is not regularly found worldwide. Its presence has been recorded in few cases in Ghana, Ivory Coast, Malaysia and Ecuador (Jespersen et al., 2005; Nielsen et al., 2007; Papalexandratou et al., 2013, 2011; Visintin et al., 2016). However, this is the first time this yeast is used as starter culture for cocoa beans fermentation as monoculture and in mixture with *Saccharomyces cerevisiae*.

Aim of this work was to study the impact of mixed cultures of *S. cerevisiae* and *T. delbrueckii* and *T. delbrueckii* monoculture on fermentation process conducted on two different cocoa hybrids. This was performed throughout studying microbiological, physico-chemical changes during the fermentation process, analyzing the volatile compounds and by sensory analysis of chocolates.

2. Materials and Methods

2.1. Starter cultures

S. *cerevisiae* IC67 and *T*. *delbrueckii* IC103 strains were isolated from spontaneous cocoa beans fermentation and they were selected as potential starter culture strains, based on previous investigations (Visintin et al., 2016). *S*. *cerevisiae* strain showed the best resistance profile for all the parameters used for screening such as temperatures, pH, as well as ethanol concentrations and tolerance to osmotic stress. Moreover, this cocoa-specific strain showed positive results for almost all the enzymatic activities, particularly β-glucosidase, glicosidase, protease and pectinase. *T*. *delbrueckii* IC103 also exhibit a suitable

profile for the tolerance screening with the only exception of ethanol concentration. Additionally, it was one of the few strains that resulted positive for esterase activity (Visintin et al., 2016).

2.2. Fermentation experiments and sampling

The fermentation experiments were conducted at the Vale do Juliana cocoa farm in Igrapiúna, Bahia, Brazil. The ripe cocoa pods from two different hybrids PS1319 and SJ02 were harvested during the main crop of 2014 (September - December). The cocoa pods were manually opened with a machete, and the beans were immediately transferred to the fermentation house. The fermentation started approximately 3 h after the breaking of the pods and was performed in 0.06 m^3 wooden boxes. Fermentations of the hybrids were performed with inoculation of hybrid PS1319 with *S*. *cerevisiae* ID67 in co-culture with *T*. *delbrueckii* ID103 and for hybrids PS1319 and SJ02 with *T*. *delbrueckii* ID103 monocultures. The inoculum was prepared in YPD broth [10 g/L Yeast extract (Merck, Whitehouse Station, NJ); 20 g/L Peptone (Himedia, Mumbai, India); 20 g/L dextrose (Merck)] at 30 °C at 150 rpm and replicated every 24 h for five days. The cells were recovered by centrifugation (7000 rpm, 10 min) and re-suspended in 1 L of sterile peptone water (1 g/L Peptone, Himedia). This solution was spread over the cocoa beans reaching a concentration of approximately 10^6 cells/g and 10^5 cells/g of cocoa for *S*. *cerevisiae* and *T*. *delbrueckii,* respectively. *S. cerevisiae* was used in higher population than *T. delbrueckii* to accelerate the fermentation process (Ramos et al., 2014), moreover the latter was inoculated in lower amounts due to the fact that is not systematically found in cocoa beans fermentation. The two hybrids were also spontaneously fermented (without inoculation). All fermentations were

evaluated for seven days, and the amount used for each assay was 300 kg of fresh beans. Samples of approximately 100 g each were collected at 0, 24, 48, 72, 96, 120, 144 and 168 h of the fermentation process. The samples were taken approximately 40 cm from the surface of the center of the fermenting cocoa mass, placed in sterile plastic pots and transferred to the laboratory. The pH values and temperatures were evaluated during fermentations by an average of three different points into the fermentation boxes using a portable pH meter Q400HM (Quimis, SP, Brazil).

2.3 Microbiological analysis

Twenty-five grams of cocoa beans and adhering pulp were added to 100 mL of peptone water (Himedia) and homogenized manually. Serial dilutions in peptone water were prepared and yeasts were enumerated by spreading on YPD agar containing 100 µg/mL chloramphenicol (Merck) in triplicate and AAB were determined by spreading on GYC agar (50 g/L glucose, 10 g/L yeast extract, 30 g/L calcium carbonate, 20 g/L agar, Merck) containing nystatin (0.4%, Merck). Plates were incubated at 30°C for 3 (yeasts) to 5 days (AAB). After the incubation time, the colonies grown in each medium were counted (mean and standard deviation were calculated). The square root of the number of colonies of each type was re-streaked and purified. The purified isolates were stored at -20°C in YPD broth containing 25% of glycerol.

2.4 DNA extraction from pure cultures

DNA was extracted from 1 ml of an overnight culture of yeasts as previously described (Cocolin et al., 2000, 2004). DNA was quantified by using the

Nanodrop Instrument (Spectrophotometer ND-1000, Thermo Fisher Scientific, Milan, Italy) and then standardized at 100 ng/μl.

2.5 (GTG)5-PCR fingerprinting

At least 5 yeast colonies for each sample at each sampling point were randomly selected, isolated and subjected to repetitive extragenic palindromic PCR (rep-PCR) to confirm the presence of starter culture. Rep-PCR was performed using the single oligonucleotide primer $(GTG)_5$ (Versalovic et al., 1994) as previously described by Dal Bello et al. (2010). The DNA profiles were compared with those of the inoculated strains through Bionumerics software, using the Dice similarity coefficient and dendrograms were obtained by means of UPGMA clustering algorithm.

2.6 Direct extraction of DNA from cocoa samples

For DNA extraction, 10 g of each sample were diluted with 10 mL of Milli-Q water, vortexed for 5 min and the supernatant collected in a Falcon tube. The step was repeated and the tubes containing 20 mL of solution were centrifuged (7000 rpm, 10 min, 4 °C), and the supernatant was separated from the precipitate. The precipitate was re-suspended in an additional 5 mL of Milli-Q water, vortexed, and centrifuged as described above. The final volume of 25 mL of diluted pulp was centrifuged and the supernatant divided from the pellet. Total DNA was extracted from 100 mg of pellet as previously described (Cocolin et al., 2000). DNA was quantified by using the Nanodrop Instrument (Spectrophotometer ND-1000, Thermo Fisher Scientific, Milan, Italy).

2.7 qPCR

Real-time PCR was carried out using the Rotor-Gene Q System (Qiagen, Hombrechtikon, ZH, Switzerland) following the method described by Batista et al. (2015). All analyses were performed in triplicate. Specific primers used in this study were previously described by Zott et al. (2010) and Diaz et al. (2013) and are as follow: SC-5fw (5'-AGGAGTGCGGTTCTTTGTAAAG-3') and SC-3bw (5'-TGAAATGCGAGATTCCCCT-3') for *S. cerevisiae*; and Tods L2 (5'-CAAAGTCATCCAAGCCAGC-3') and Tods R2 (5'-TTCTCAAACAATCATGTTTGGTAG-3') for *T. delbrueckii*. For standard curves, *S. cerevisiae* and *T. delbrueckii* starter culture were cultivated in YPD agar at 30°C for 24 h. The cells were counted using a Neubauer chamber and DNA was extracted as previously described and serially diluted (1:10) from 10^8 and 10^7 for *S. cerevisiae* and *T. delbrueckii* respectively, down to 10^2 cell/mL. Each point on the calibration curve was measured in triplicate.

2.8 Metabolites extraction and HPLC analyses

The carbohydrates, alcohols and organic acids were extracted from 10 grams of cocoa beans of each sample as described by Rodriguez-Campos et al. (2011) and successively modified by Ramos et al. (2014). The final volume of 25 mL of diluted pulp was centrifuged, and 2 mL of supernatant was filtered through a 0.22 μm membrane (Millipore) for the HPLC analysis. After the pulp extraction the cocoa beans were deprived of the testa and crushed using a pestle and mortar. The carbohydrates (glucose and fructose), organic acids (acetic, lactic and citric acids) and alcohol (ethanol) analyses were then extracted using a liquid chromatography system (Shimadzu, model LC-10Ai, Shimadzu Corp., Japan) provided with a dual detection system: a UV–Vis detector (SPD10Ai) for the detections of acids group (210 nm) and a refractive index detector (RID-

10Ai) for alcohols and carbohydrates as described by Batista et al. (2015). AShimadzu ion exclusion column (Shim-pack SCR- 101H, 7.9 mm× 30 cm) was set up at 50 °C. Perchloric acid (100 mM) was used as the eluent at a flow rate of 0.6 mL/min. Retention time of the standards injected using the same conditions were used to identify the compounds. The sample concentrations were calculated using an external calibration method, while the calibration curves were obtained by injecting different concentrations of the standards at the same conditions of the samples. Each area was successively plotted in a linear curve in order to estimate the concentration of the compounds. Standard (purity N 99.8%), glucose, fructose, and citric acid were purchased from Sigma-Aldrich (Saint Luis, EUA); acetic acid and ethanol, were bought from Merck (Darmstadt, Germany); and lactic acid from Fluka Analyticals (Seelze, Germany).

2.9. Chocolate preparation and analyses

The dried beans were sent for chocolate production at Sartori and Pedroso Alimentos Ltda. (Sao Roque, SP, Brazil). The chocolate was prepared with 70% cocoa.

2.9.1 Volatile compounds extraction and GC-MS

The volatile compounds of the chocolate samples (2.0 g) were extracted using the solid phase micro extraction technique in the headspace (SPME-HS). Samples were transformed into a fine powder with a grinder (IKA A11 Basic analytical mill) using liquid nitrogen and transferred to a sealed vial.

A 50/30 μm divinylbenzene/carboxene/polydimethylsiloxane (DVB/CAR/PDMS) fiber provided by Supelco was used to extract volatile compounds. The fiber

was balanced for 15 min at 60°C and then exposed to the cocoa powder for 30 min at the same temperature. The compounds were analyzed using a Shimadzu GCMS QP2010SE model equipped with a capillary column of silica Carbowax 20M (30 m × 0.25 mm i.d. × 0.25 µm) (J & W Scientific, Folsom, CA, USA). The temperature program began with 5 min at 60°C, followed by a gradient of 60°C to 230°C at 10°C/min; the temperature was then maintained at 230°C for 15 min. The carrier gas (N_2) was used at a flow rate of 1.95 mL/min. Injections were performed by fiber exposition for 2 min. Volatile compounds were identified by comparing the retention times of the compounds in the samples with the retention times of standard compounds injected under the same conditions. Quantitative data of the identified compounds were obtained by integrating the peak areas of all identified compounds. The relative percentages of individual compounds were calculated from the total contents of volatiles on the chromatograms. All samples were examined in duplicate.

2.9.2 Sensory analysis

Sensory analyses of samples made from controls and inoculated fermentations were performed using a consumer acceptance test followed by a check-all-that-apply (CATA) question. The tests were conducted on 71 adults over 18 years of age, 41% male and 59% female, regular consumers of dark chocolate. For the acceptance test, the consumers evaluated how much they liked each sample using a 9-point hedonic scale with 1 = dislike extremely and 9 = like extremely (Stone and Sidel, 1993). For the CATA question, the consumers were asked to evaluate seven sensory attributes and select those they considered appropriate to describe the chocolate. The attributes were acid flavor, astringent, banana, desirable bitter, undesirable bitter, buttery, chestnut flavor, coffee flavor, citrus

flavor, fruity, earthy flavor, peanut flavor, rancid, roast, sweet, vanilla flavor and woody. The tests were performed as previously described by Batista et al. (2015)

2.10 Statistical analyses

Data obtained from pH, temperatures and counts, as well as GC-MS data were analyzed using a one-way analysis of variance (ANOVA). Results showing significance variance (p<0,05) were subjected to a post-hoc Duncan comparison to identify significantly different samples. While data obtained from sensory analysis were analyzed using a Friedman test and a post-hoc paired Wilcoxon rank sum test, to check for significant differences in the profiles. Analyses were performed with IBM SPSS 21.1 software package.

3. Results

3.1 Microbiological analysis of spontaneous and inoculated fermentations

The temperature, pH and counts of yeasts and AAB during PS1319 and SJ02 spontaneous and inoculated fermentations are reported in Tables 1A and 1B, respectively. All fermentations lasted 192 hours, except PS1319 inoculated with the mixture of *S. cerevisiae* and *T. delbrueckii,* which lasted 168 h.

The temperature values were significantly different (p<0.001) between PS1319 inoculated fermentations and the control one, at all sampling times except at 192 hours. At 48 and 96 hours, the temperature of PS1319 inoculated with *S. cerevisiae* and *T. delbrueckii* was significantly higher (30.3±0.2 and 46.4±0.1°C, respectively) with respect to PS1319 inoculated only with *T. delbrueckii* and to

the control (25.8±0.9 and 25.1±0.2°C; 30.7±0.3 and 41±0.2°C respectively). Differences were recorded at 24, 72, 96, 120 and 192 hours for pH values (p<0.01 and p<0.05).

Significant differences were also observed within PS1319 inoculated with *S. cerevisiae* and *T. delbrueckii* and control fermentations at 0, 24, 48, 72, 96 and 168 hours in the yeast counts (p<0.0001, Tab. 1A) and during all the fermentations in the case of the AAB counts (p<0.001 and p<0.01). Yeast counts were significantly lower in PS1319 control (values ranging from 4.2±0.2 to 5±0 Log_{10} cfu/g) with respect to the inoculated boxes for the first 96 hours. In all three cases a strong decrease was observed later in the process. AAB counts were significantly higher in PS1319 experiment inoculated with *S*. *cerevisiae* and *T*. *delbrueckii* (4±0, 5.5±0, 7±0 and 7±0 Log_{10} cfu/g) with respect to PS1319 inoculated with *T*. *delbrueckii* and the control one at 96, 120, 144 and 168 hours, as reported in Table 1A.

Concerning SJ02 hybrid fermentations, significant differences were observed after 24 hours for pH values and at 0 and 72 hours for temperature measures (p<0.001). Furthermore, significant differences were recorded between inoculated and spontaneous fermentations at 0, 72, 96, 120 and 168 hours in the yeast counts (p<0.001, Tab. 1B) and during almost all the process for AAB counts (p<0.001 and p<0.01), except for 96 and 192 hours. Although starter culture and consequently the microbiota present in the different assay affect directly the parameters such as pH and temperature, variations may also be related to external parameters e.g. environmental temperature and microbial contamination. The assays were performed in farm scale and these parameters

could not be controlled, however all assay were performed at same time and subject to the same conditions.

3.2 Quantification of *S. cerevisiae* and *T. delbrueckii* by qPCR analysis

Dynamic changes of the population of the two starter culture species during cocoa fermentations were monitored by qPCR. Standard curves were established for each primer set, correlation coefficient of 0.998 and 0.999 and efficiency reaction values of 97 and 94 % were obtained for *S. cerevisiae* and *T. delbrueckii* primers, respectively. The lowest detection limit was 10^2 cells mL^{-1}. *S. cerevisiae* and *T. delbrueckii* were detected and quantified during inoculated and spontaneous fermentations as shown in Table 2.

Concerning PS1319 experiments, the population of *S. cerevisiae* was lower in the control, ranging from 3 to 6.4 log cfu/g, than in the inoculated fermentation (inoculated with *S*. *cerevisiae* and *T*. *delbrueckii*), ranging from 4.4 to 6.7 log cfu/g, except at 120 hours. In the control, *T. delbrueckii* was almost not detected as reported in Table 2. In PS1319 inoculated with *S*. *cerevisiae* and *T*. *delbrueckii*, *T. delbrueckii* population ranged from 4.7 to 5 log cfu/g in the first 48 hours and then decreasing to 3 to 2.6 log cfu/g during the last phase. Quantification of *T. delbrueckii* in PS1319 (inoculated only with *T*. *delbrueckii*) resulted in 4.7 log cells/g and 6 log cells/g after 24, 48 h and 72 hours, respectively.

During SJ02 cocoa bean fermentations, *T. delbrueckii* ranged from 3.7 to 5.5 log cell/g, whereas in the control assay it was almost not detected (2 to 2.3 log cfu/g).

3.3 Implantation of starter cultures

$(GTG)_5$-PCR fingerprinting was used to type 157 isolates (randomly selected) at strain level allowing to confirm the implantation of the starter cultures strains. The cluster analysis of the profiles obtained by Rep-PCR for PS1319 inoculated with *S*. *cerevisiae* and *T*. *delbrueckii*, and PS1319 and SJ02 inoculated with *T*. *delbrueckii* experiments are shown in Figures 1A, 1B and 1C, respectively. In all the cluster analyses, groups showing the same bands profile as *S. cerevisiae* and *T. delbrueckii* strains, added to the respectively inoculated batches, were distinguished. Indeed, in the case of PS1319 inoculated with the mixed culture, at day 0, 94% of the isolates grouped as *T. delbrueckii* profile, while *S. cerevisiae* represented 1% of the isolates, increasing up to 100% at 48 and 96 h, while at day 3 and 4, *S. cerevisiae* was present with 75 and 50% of the isolates, respectively (Fig. 1A). *T. delbrueckii* inoculated in PS1319 in monoculture corresponded to 91, 25 and 20 % at days 0, 1 and 2, respectively (Fig. 1B). In case of SJ02 monoculture experiment, *T. delbrueckii* was present at day 0 with a total of 100% of the profiles (Fig. 1C).

Isolates of those main clusters showed a similarity of 95 to 100% as reported in Figures 1A, 1B and 1C. Clusters of *T. delbrueckii* profiles were composed only of strains isolated at day 0, in case of PS1319 inoculated with *S*. *cerevisiae* and *T*. *delbrueckii* and SJ02 inoculated with *T. delbrueckii* only (Fig. 1A and 1C), while during PS1319 fermentation inoculated only with *T*. *delbreueckii*, strains of *T. delbrueckii* were isolated at day 0, 1 and 2. On the other hand, *S. cerevisiae* profiles were observed from 0 to 5 days in PS1319 inoculated with *S*. *cerevisiae* and *T*. *delbrueckii* box.

T. delbrueckii was only isolated at the beginning of the process and never found after 2 days of fermentation. This is in agreement with the poor competitive abilities of this species in mixed yeasts ecosystems (Bely et al., 2008).

3.4 Chemical changes during fermentations

Composition in organic acids (acetic, lactic and citric acids), sugars (glucose and fructose) and alcohols (ethanol) of the cocoa beans samples analyzed is reported in Figure 2.

Figures 2A, 2C, 2E, 2G and 2I show the results obtained from the analysis of the pulp. Carbohydrates were consumed faster in the fermentation inoculated with *S. cerevisiae* and *T. delbrueckii* than in the control for PS1319 trials (Fig. 2A and 2E). Inoculated experiments showed the faster consumption of citric acid in the first hours as shown in Figure 2. Ethanol concentration in the pulp was higher in the PS1319 fermentation inoculated with yeasts, reaching the peak of ethanol production (around 7 g/kg) at 72 h and 96 h for boxes inoculated with *S. cerevisiae* and *T. delbrueckii,* respectively, compared to the control (Fig. 2E). SJ02 hybrids fermentations showed the same value of 6.01 g/kg of ethanol at 72 hours. For the acetic acid, the maximum concentrations were higher between SJ02 inoculated fermentations and the control: 1.8 e 1.0 g/kg, respectively. Different trends were observed for lactic acid within the samples as reported in Figure 2A, 2C, 2E, 2G and 2I.

Figures 2B, 2D, 2F, 2H and 2L show the concentration of carbohydrates, ethanol and organic acids in the beans. The concentration of carbohydrates was similar in all the unfermented cocoa beans as shown in Figure 2. Citric acid was the higher detected compound present in all the experiments, except for

SJ02 inoculated box. A peak of lactic acid was observed between 144 and 168 hours (Fig. 2B, 2D and 2L) and at the end of the process (Fig. 2F). The highest concentration of acetic acid was recorded within 144 and 168 hours. (Fig. 2B, 2D and 2F).

3.5 Volatile compounds

SPME-GC/MS method enabled the identification of 36 compounds in PS1319 chocolate samples and 34 in SJ02 trials, as shown in Table 3, respectively. These compounds were mainly grouped into acid, alcohols, aldehydes and ketones, esters and lactones. Within PS1319 clone experiments, significant differences among these classes (except for pyrrole compound) were noticed. On the contrary, no significant differences within SJ02 inoculated with *T. delbrueckii* and the control on the volatile compounds were observed. Alcohols was the main group of volatile compounds detected.

Within the acids, hexanoic, isovaleric and 3-hydroxy-2- methyl-4-pyrone acids were found to be significantly different ($p<0.05$) among PS1319 experiments. Content of 2-phenylacetic acid was higher in chocolates made from *T. delbrueckii* inoculated cocoa beans clones. For what concerns alcohols group, statistical analysis showed a significantly higher ($p<0.001$) 2-phenylethanol content in PS1319 inoculated fermentations with respect to the control one.

GC-MS data showed significantly higher amounts of aldehydes and ketones compounds in chocolate made from PS1319 inoculated with *T. delbrueckii* (16.1±0.7%*)* with respect to the others, as reported in Table 3. Within this group 2-acetylpyrrole and acetoin resulted as the most impacting, as reported in Table 3. Statistically different amounts of solerone was detected in chocolates made with PS1319 inoculated with *S. cerevisiae* and *T. delbrueckii* (1.8±0.2%) and

PS1319 inoculated with *T. delbrueckii* (0.7±0%) while it was not found in the control sample.

3.6 Sensory evaluation of chocolate

Sensory analyses allowed to differentiate between the chocolates produced from spontaneous and inoculated fermentation. Data of the overall acceptance of the 5 chocolates, judged by the sensory panel, resulted as being significantly different (p<0.001) by the Friedman test. SJ02 inoculated with T. *delbrueckii* was the most appreciated within the samples (scored 7.3 on the 9-point edonic scale), however the overall acceptance showed that it was not significantly different from SJ02 control (scored 7.1), PS1319 inoculated with *S. cerevisiae* and *T. delbreuckii* (scored 7) resulted significantly more acceptable by the post-hoc analysis (p<0.001) compare to PS1319 spontaneous fermentation (scored 6.2).

The evaluation of the seventeen parameters used by the sensory panel to describe the sensory profile of chocolate was significantly different (p<0.001). Answers to the CATA questions highlighted that the main parameters used to describe the chocolates were desirable bitter, sweet and coffee flavor. SJ02 inoculated with *T*. *delbrueckii*, as reported in Figure 3B, was described as fruitier and more roasted than the control, on the contrary SJ02 was more sweet, with a lower frequency of undesirable bitter taste and less acid then the inoculated one. Figure 3A shows CATA results for chocolate profiles of PS1319 fermentations. Inoculated fermentations resulted in a chocolate with higher values of desirable bitter taste, sweet, coffee flavour, fruity and roast. Moreover, PS1319 inoculated with *S. cerevisiae* and *T. delbreuckii* showed a reduction in astringent, woody, undesirable taste of bitter and hearty flavour.

4. Discussion

The aim of this work was to study the impact of mixed cultures of *S. cerevisiae* IC67 and *T. delbrueckii* IC103 and *T. delbrueckii* monoculture on fermentation processes conducted on two different hybrids, PS1319 and SJ02, in Bahia, Brazil. Ramos et al. (2014) recently described how diverse hybrids with different characteristics and different initial substrates (carbohydrates and citric acid) influence the microbial profile and the volatile compounds, during the fermentation process. While *S. cerevisiae* has been repeatedly defined as one of the most important yeasts involved in cocoa beans fermentations, as recently reviewed by Schwan and Fleet (2014), *T*. *delbrueckii* was not regularly found. IC103 strain was characterized for its enzymatic activities (Visintin et al., 2016) that may have an important impact on chocolate aroma profile. Indeed, esterase, β-glucosidase and glicosidase made *T*. *delbrueckii* IC103 strain to be selected as suitable cocoa beans fermentation starter culture. *T*. *delbrueckii* has been frequently described as an important yeast in wine sector showing high fermentation ability with production of very low levels of volatile acidity and acetaldehyde, which has been positively perceived as contribute to the flavor of alcoholic beverages (Bely et al., 2008; Ciani and Maccarelli, 1998). Moreover, some strains of *T*. *delbrueckii* have been commercialized and are available to the winemaking industry.

The yeast metabolism results in ethanol production, which will be transformed to acetic acid by AAB increasing the temperature during the fermentation with a consequent perceptible strong vinegar-like aroma. Furthermore, microbial activities consume the citric acid present in the pulp causing an increase in pH.

Temperature and pH changes were detected for all different fermentations performed in the present study, however some assays showed greater and/or faster changes than others. *S*. *cerevisiae* and *T*. *delbrueckii* inoculation seemed to accelerate the temperature and pH increase for the hybrid PS1319. PS1319 and SJ02 hybrids inoculated with *T*. *delbrueckii* showed a slower temperature raise compare to the spontaneous processes. Inoculated cocoa bean fermentations were characterized by higher counts of yeasts during the initial phase. Ethanol produced by *S*. *cerevisiae* and *T*. *delbrueckii* seemed to have influenced AAB fermentation, indeed AAB reached higher concentration faster than other PS1319 trials performed in this study. In the present study LAB were not considered due to the fact that recently it has been hypothesized that LAB may not be necessary for a successful cocoa fermentation. Ho et al. (2015) reported that beans fermented in the presence or absence of LAB were equally fully fermented, showing similar shell weights and acceptable chocolates with no differences in sensory rankings.

Quantitative PCR underlined a higher amount of *S*. *cerevisiae* and *T*. *delbrueckii* populations in the inoculated boxes, as expected. The counts determined might be even higher if we consider a possible matrix effects that may have caused a delay in cycle measurement and thereof an underestimation of the counts. The use of qPCR for complex matrix has already been validated (Schwendimann et al., 2015; Zott et al., 2010), and this specific approach has already been previously used in other studies (Batista et al., 2015; Menezes et al., 2016). Quantification data of *T*. *delbrueckii* during spontaneous fermentation confirmed the attitude of this species of not being so commonly involved in this process. Results of $(GTG)_5$-PCR fingerprinting

allowed us to evaluate the implantation of *S*. *cerevisiae* and *T*. *delbrueckii* used as starter culture at strain levels. Cluster analysis results were in agreement with *T*. *delbrueckii* expected behavior, since clusters were composed by strains isolated during the first 24 hours of fermentation. On the contrary, *S*. *cerevisiae*, the most strongly fermenting ethanol-tolerant species, took over the fermentation at the second and the fifth day.

Microbial activity generated metabolites and conditions (temperature and pH) that killed the beans, thereby causing an array of biochemical reactions and chemical changes within the bean itself, that are essential for the development of the complex flavor of chocolate (Pereira et al., 2012). As likely, carbohydrates (glucose and fructose) and citric acid consumption were observed at the initial times of fermentation (until around 72 h) and it was faster in the PS1319 fermentation inoculated with *S*. *cerevisiae* and *T*. *delbrueckii* than in others. In agreement with a slower fermenting ability of *T*. *delbrueckii*, at the beginning of SJ02 fermentations (24 h), the control showed a faster consumption in sugars with respect to the inoculated one, wherein a delay in the acetic acid production was also probably correlated. In agreement with a higher amount of cells, during PS1319 mixed experiment, an increased amount of acetic acid was recorded.

Some of the herein detected volatile compounds have been reported as responsible for producing desirable note flavors and off flavors in cocoa beans during the fermentation, drying and roasting processes. For examples, alcohols such as 2,3-butanediol and 2-phenylethanol have been reported in cacao fermentation and these compounds are desirable for high quality cocoa products (Schwan and Wheals, 2004). On the contrary, GC-MS also found

small amounts of propanoic, hexanoic, 1-octanoic, 1-nonanoic and 1-dodecanoic acids in chocolate samples, which may have a negative effect on cocoa aromatic quality. To note the lack of detection of pyrazines, believed to be relevant for the chocolate flavor (Afoakwa et al., 2008). The presence of pyrazines can be influenced by different parameters such as the type and time of fermentation, the wether condition, the ripeness of pod, the type of cocoa, variations in pulp/bean percentage and the storage of pods. Moreover, the chocolate processing can influence the development of pyrazines

A good correlation between the volatile aroma profile of inoculated and spontaneous PS1319 and SJ02 fermentations and the sensory perception of the chocolates appeared to exist. Since chocolate is a complex matrix, a precise link between volatile compounds and specific sensory attributes is difficult. Nevertheless, the cocoa flavor perceived by the tasters in the PS1319 *T*. *delbrueckii* inoculated chocolate correlated well with the high concentrations of Strecker aldehydes measured by GC–MS with respect to the chocolate made by cocoa beans from spontaneous fermentations.

Judges described the chocolates made from beans fermented with the use of starter cultures as being fruity, which corresponds well with the *S*. *cerevisiae* and *T*. *delbreuckii* inoculated chocolate having the highest concentration of 2-acetylpyrrole, phenethyl acetate and 2-phenylethanol compounds. Particularly, PS1319, inoculated with *T*. *delbreueckii,* chocolate profile was the one that showed highest values of likely aroma compounds as 2-phenylacetic acid, furfuryl alcohol, 2-acetylpyrrole and acetoin (butanoate metabolism). Within these compounds some are well known coming from yeast metabolism such as phenethyl acetate, 2-phenylethanol, 2-phenylacetic acid and acetoin. No

significant differences in the volatile compounds were noticed within inoculated and spontaneous SJ02 fermentation chocolates, this is in agreement with overall acceptance of sensory analysis, whereas few differences have been observed by CATA test. Fruity and roast descriptors have been used with more frequency respect the control, on the contrary this last was described as more sweet, with a lower frequency of undesirable bitter taste and less acid than the inoculated one.

The results showed that the consumers were able to differentiate the samples. Flavor characteristic of chocolate is determined by the genetically potential of the cocoa bean variety, how the steps of fermentation and drying are carry out, and how the chocolate processing stages are applied by the manufactures (Afoakwa et al., 2008; Crafack et al., 2014). As well, fermentation is considered by the literature to be the most important factor influencing the flavor quality of cocoa and thereof on chocolate. More than 600 volatile compounds are reported to mark up the complex mixture that characterizes chocolate aroma (Counet et al., 2002). An appropriately conducted fermentation safeguards liberation of flavor precursors that are necessary for the formation of aroma compounds, but also influence the value of acids, alcohols, esters and ketones produced by yeasts, LAB and AAB during the fermentative process (Afoakwa et al., 2008; Lima et al., 2011; Schwan and Wheals, 2004).

This study shows that *S. cerevisiae* ID67 and *T. delbrueckii* ID103 starter cultures were able to grow and succeed during cocoa hybrids fermentations. Moreover, they influenced the PS1319 hybrid end-product quality changing analytic profile and sensory perception of chocolates. No big influenced were recorded for SJ02 hybrid, but this may be improved by using a higher starter

inoculations or a co-culture with *S. cerevisiae*. In particular, in combination with the *S*. *cerevisiae* strain, *T*. *delbrueckii* had a positive influence on the analytical profile of chocolates. The application of starter cultures did change the aroma profile of the resulting chocolate as determined by GC–MS, in some cases the differences observed were too small to significantly change consumer perception of the chocolates, but in other cases they had a significantly impact on those. Recently studies using yeasts starter cultures report successful changes in aroma profile with respect to spontaneous fermentation (Batista et al., 2016; Meersman et al., 2016; Ho et al., 2014). The different results between SJ02 and PS1319 experiments suggest that the use of these starter culture strains may be appropriated for certain cocoa varieties but not for all, as also observed by Menezes et al. (2016).

The evaluation of *T*. *delbrueckii* and *S*. *cerevisiae* yeasts starter used in the present study might be of interesting value for the cocoa world to enhance the quality, improve the complexity, and modify some of parameters of the final end-product.

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Figure legends

Figure 1 Dendrogram obtained by cluster analysis of PS1319 inoculated with *S. cerevisiae* and *T. delbrueckii* (A), PS1319 inoculated with *T. delbrueckii* (B) and SJ02 inoculated with *T. delbrueckii* (C) yeast isolates. The groups, obtained by means of cluster analysis of the Rep-PCR profiles, were calculated using Dice correlation index. Strain code, with the respective day of isolation, is reported. S1 and U1 in the strain code refer to *S. cerevisiae* ID67 and *T. delbrueckii* ID103 inoculated strains, red and green lines indicate *S. cerevisiae* and *T. delbrueckii* isolates groups respectively.

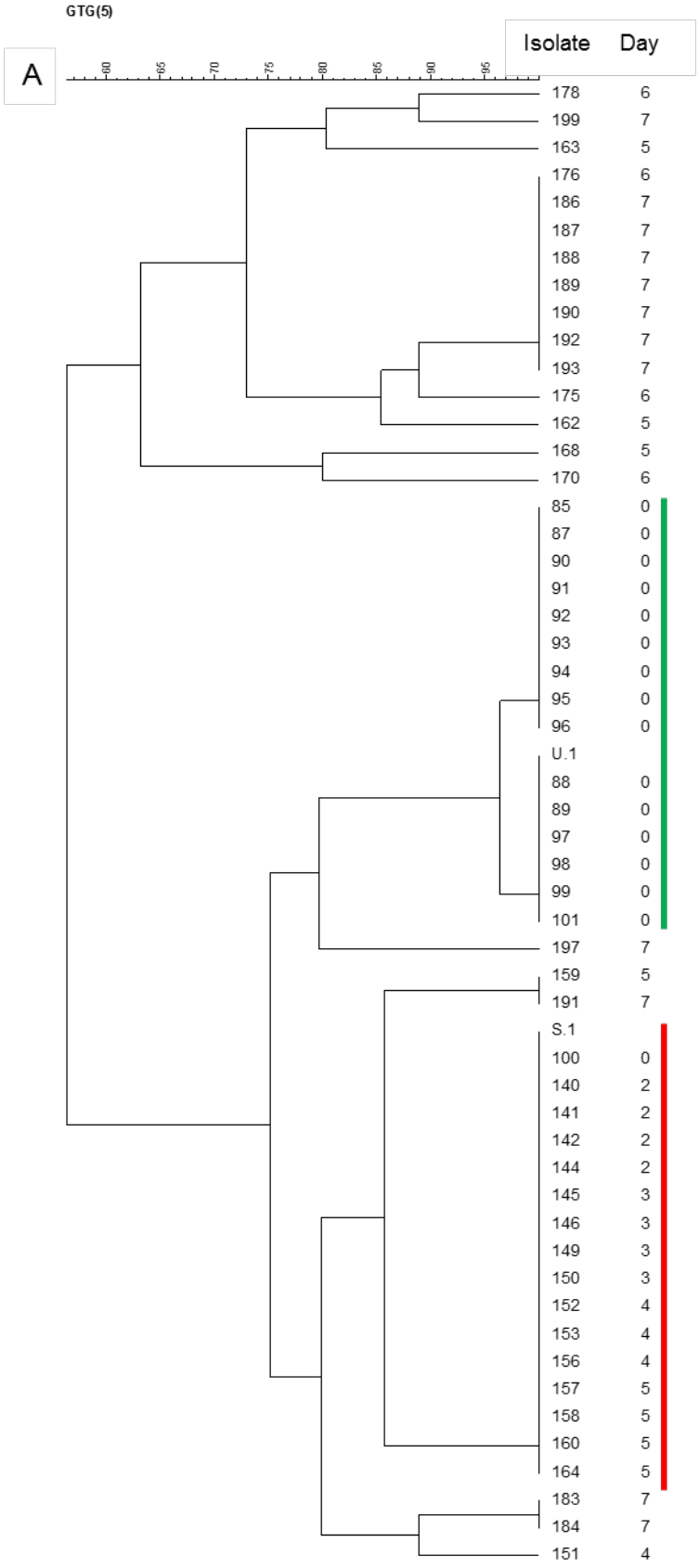
Figure 2 Changes of carbohydrates (glucose and fructose), alcohol (ethanol) and organic acids (acetic, lactic and citric acids) detected in the pulp (A, C, E, G, I) and in the beans (B, D, F, H, L) during spontaneous and inoculated fermentations. Fig 2A and 2B correspond to PS1319 fermentation inoculated with *S. cerevisiae* ID67 and *T. delbrueckii* ID103; Fig. 2C and 2D refer to PS1319 conducted with *T. delbrueckii* ID103*;* Fig. 2E and 2F mean PS1319 control; 2G and 2H correspond to SJ02 conducted with *T. delbrueckii* and 2I, 2L to SJ02 spontaneous fermentations. Data are expressed as concentration of g/kg.

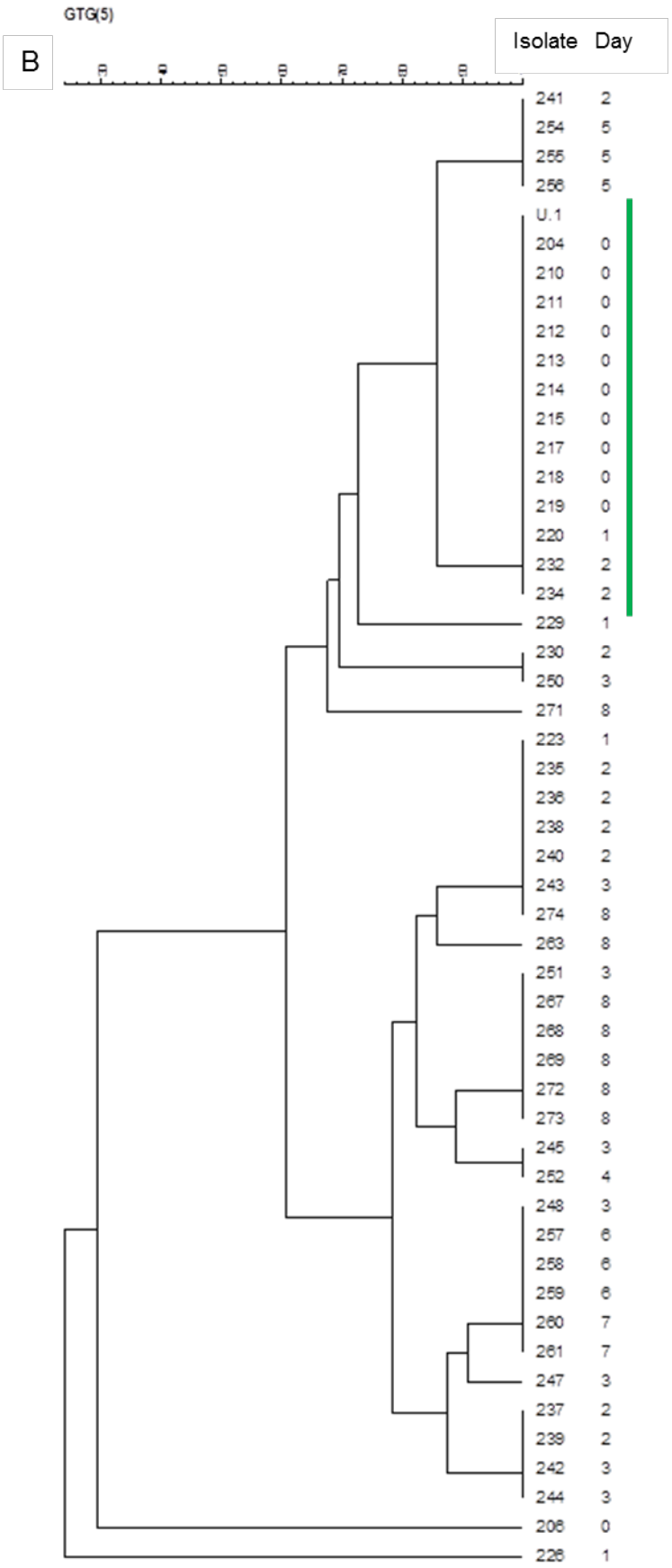
Figure 3 Aroma profiles of the chocolates produced from PS1319 (A) and SJ02 (B) cocoa beans fermented with inoculations of yeasts starter culture (PS1319 ST, PS1319 T and SJ02 T) and cocoa beans spontaneously fermented

(PS1319 and SJ02). Fermentation with ST code means inoculated with *S. cerevisiae* ID67 and *T. delbrueckii* ID103; and T with *T. delbrueckii* ID103.

The center of the diagram corresponds to the lowest flavor intensity and the perimeter to the highest flavor intensity.

Figure 1


GTG(5)
A
60
65
70
75
80
85
90
95
Isolate
Day
178 6
199 7
163 5
176 6
186 7
187 7
188 7
189 7
190 7
192 7
193 7
175 6
162 5
168 5
170 6
85 0
87 0
90 0
91 0
92 0
93 0
94 0
95 0
96 0
U.1
88 0
89 0
97 0
98 0
99 0
101 0
197 7
159 5
191 7
S.1
100 0
140 2
141 2
142 2
144 2
145 3
146 3
149 3
150 3
152 4
153 4
156 4
157 5
158 5
160 5
164 5
183 7
184 7
151 4


B
GTG(5)
Isolate Day
241 2
254 5
255 5
256 5
U.1
204 0
210 0
211 0
212 0
213 0
214 0
215 0
217 0
218 0
219 0
220 1
232 2
234 2
229 1
230 2
250 3
271 8
223 1
235 2
236 2
238 2
240 2
243 3
274 8
263 8
251 3
267 8
268 8
269 8
272 8
273 8
245 3
252 4
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257 6
258 6
259 6
260 7
261 7
247 3
237 2
239 2
242 3
244 3
206 0
226 1

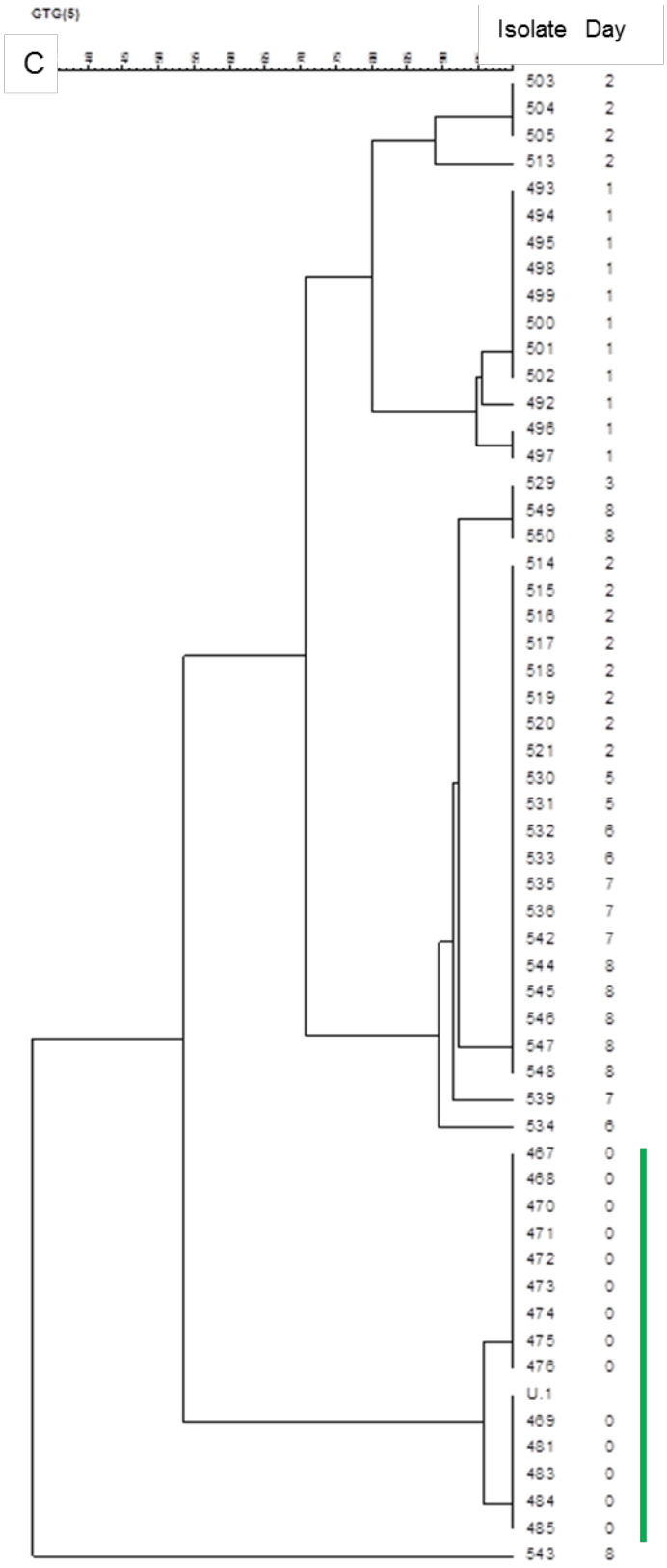
GTG(5)
C
Isolate Day
503 2
504 2
505 2
513 2
493 1
494 1
495 1
498 1
499 1
500 1
501 1
502 1
492 1
496 1
497 1
529 3
549 8
550 8
514 2
515 2
516 2
517 2
518 2
519 2
520 2
521 2
530 5
531 5
532 6
533 6
535 7
536 7
542 7
544 8
545 8
546 8
547 8
548 8
539 7
534 6
467 0
468 0
470 0
471 0
472 0
473 0
474 0
475 0
476 0
U.1
469 0
481 0
483 0
484 0
485 0
543 8

Figure 2

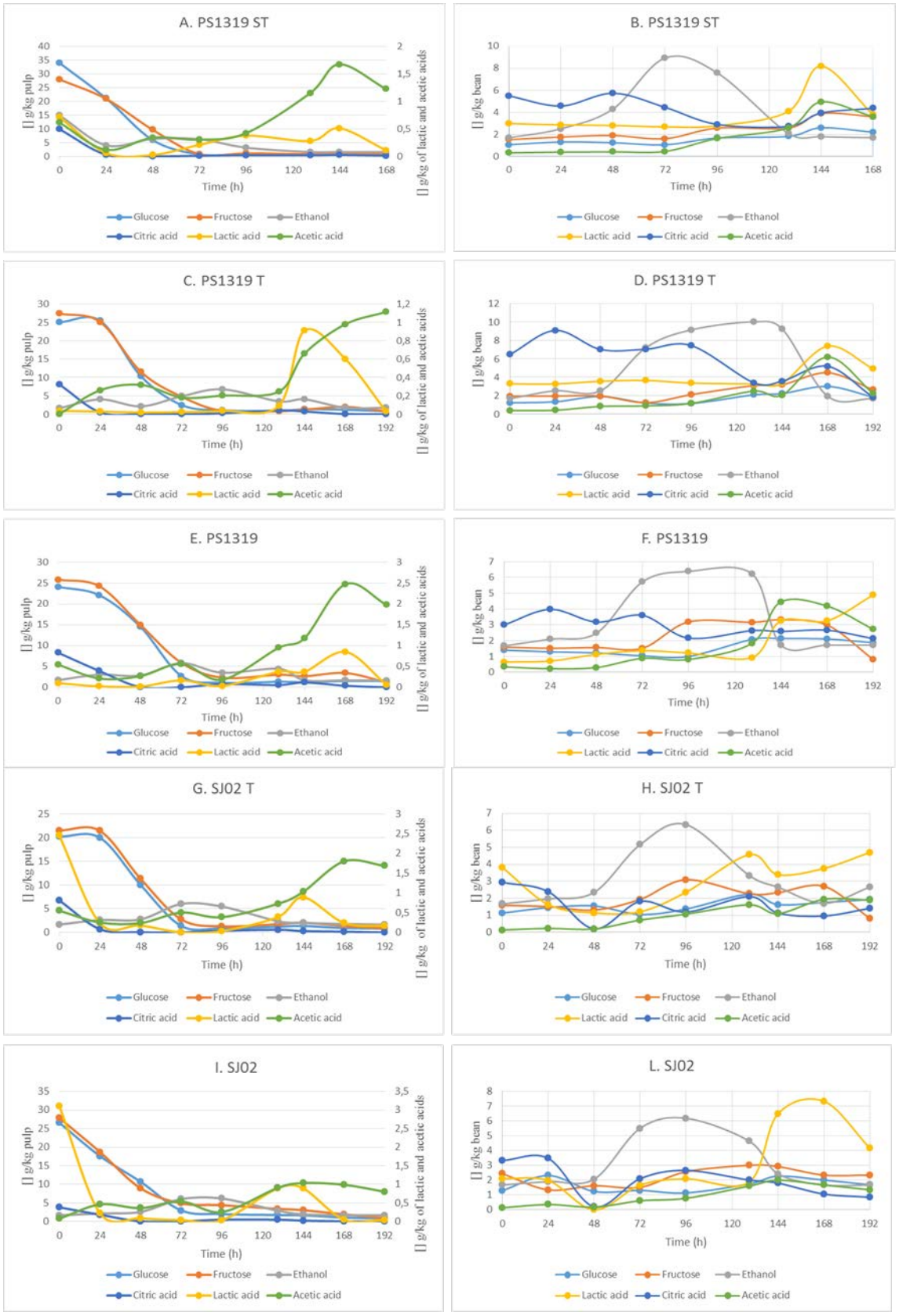
A. PS1319 ST
[] g/kg pulp
[] g/kg of lactic and acetic acids
Time (h)
Glucose
Fructose
Ethanol
Citric acid
Lactic acid
Acetic acid
B. PS1319 ST
[] g/kg bean
Time (h)
Glucose
Fructose
Ethanol
Lactic acid
Citric acid
Acetic acid
C. PS1319 T
[] g/kg pulp
[] g/kg of lactic and acetic acids
Time (h)
Glucose
Fructose
Ethanol
Citric acid
Lactic acid
Acetic acid
D. PS1319 T
[] g/kg bean
Time (h)
Glucose
Fructose
Ethanol
Lactic acid
Citric acid
Acetic acid
E. PS1319
[] g/kg pulp
[] g/kg of lactic and acetic acids
Time (h)
Glucose
Fructose
Ethanol
Citric acid
Lactic acid
Acetic acid
F. PS1319
[] g/kg bean
Time (h)
Glucose
Fructose
Ethanol
Lactic acid
Citric acid
Acetic acid
G. SJ02 T
[] g/kg pulp
[] g/kg of lactic and acetic acids
Time (h)
Glucose
Fructose
Ethanol
Citric acid
Lactic acid
Acetic acid
H. SJ02 T
[] g/kg bean
Time (h)
Glucose
Fructose
Ethanol
Lactic acid
Citric acid
Acetic acid
I. SJ02
[] g/kg pulp
[] g/kg of lactic and acetic acids
Time (h)
Glucose
Fructose
Ethanol
Citric acid
Lactic acid
Acetic acid
L. SJ02
[] g/kg bean
Time (h)
Glucose
Fructose
Ethanol
Lactic acid
Citric acid
Acetic acid

Figure 3

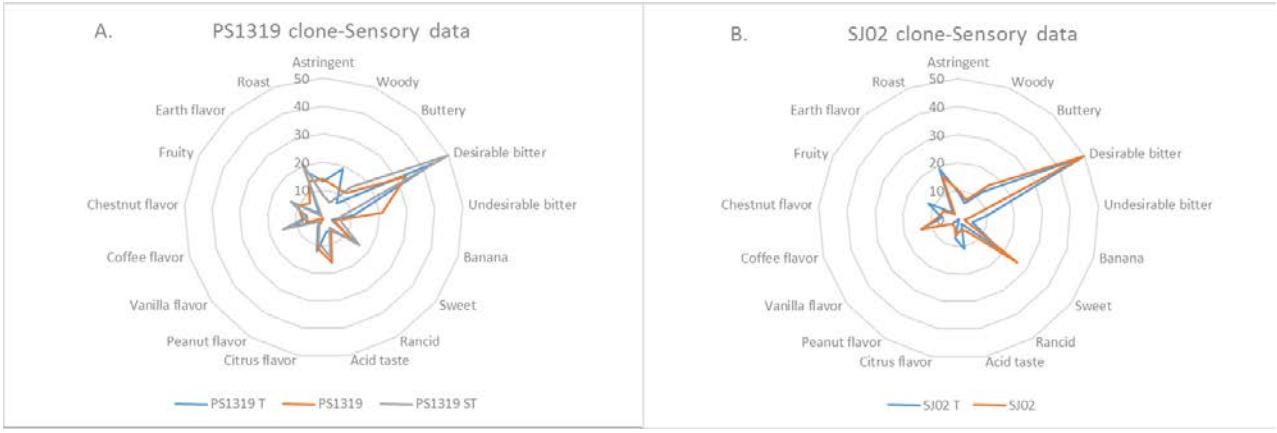
A.
PS1319 clone-Sensory data
Astringent
Woody
Buttery
Desirable bitter
Undesirable bitter
Banana
Sweet
Rancid
Acid taste
Citrus flavor
Peanut flavor
Vanilla flavor
Coffee flavor
Chestnut flavor
Fruity
Earth flavor
Roast
50
40
30
20
10
PS1319 T
PS1319
PS1319 ST
B.
SJ02 clone-Sensory data
Astringent
Woody
Buttery
Desirable bitter
Undesirable bitter
Banana
Sweet
Rancid
Acid taste
Citrus flavor
Peanut flavor
Vanilla flavor
Coffee flavor
Chestnut flavor
Fruity
Earth flavor
Roast
50
40
30
20
10
SJ02 T
SJ02

Table legends

Table 1 pH, temperature measurements, yeast and AAB counts during spontaneous and inoculated PS1319 (A) and SJ02 (B) hybrids fermentations. ST means inoculated with *S. cerevisiae* ID67 and *T. delbrueckii* ID103; and T with *T. delbrueckii* ID103. The values of pH, T° and CFU are the mean ± standard deviation of three analyses.

Statistical data are expressed as *, **, *** and NS that indicate significance at $p<0,05$, $p<0,01$ and $p<0,001$ and no significance, respectively. Different letters $^{a\text{-}c}$ within the same fermentation time and the same parameters are significantly different ($p<0,05$). na means not applicable.

Table 2 Results of quantification of *S. cerevisiae* and *T. delbrueckii* populations during inoculated PS1319 and SJ02 hybrids fermentations by qPCR. Fermentation code: ST means inoculated with *S. cerevisiae* ID67 and *T. delbrueckii* ID103; and T with *T. delbrueckii* ID103. The concentration values expressed as Log CFU/g are mean ± standard deviation of three replicates.

* below quantification limit

na, not applicable.

Table 3 Concentration of volatile compounds obtained by GC-MS analysis of chocolate produced from spontaneous and inoculated PS1319 and SJ02 hybrids fermentations. In sample code ST means inoculated with *S. cerevisiae* ID67 and *T. delbrueckii* ID103; and T with *T. delbrueckii* ID103. The values are

the mean ± standard deviation of two determinations. Data are expressed as relative percentage from total area of the peaks.

Statistical data are expressed as *, **, *** and NS that indicate significance at $P<0,05$, $P<0,01$ and $P<0,001$ and no significance, respectively. Different letters $^{a-c}$ within fermentations of the same hybrids indicate that are significantly different ($P<0,05$).

Table 1

A	Fermentation code	Fermentation time (h)								
		0	24	48	72	96	120	144	168	192
pH	PS1319 ST	3.4±0.2	3.4±0.1^{a}	4±0.2	4.4±0.1^{b}	4.8±0.2^{b}	4.4±0.1^{a}	4.2±0.3	4.4±0.2	na
	PS1319 T	3.4±0.1	3.7±0.1^{b}	4±0.2	4±0.1^{a}	3.7±0.3^{a}	4.7±0.1^{b}	4.7±0.3	4.3±0.1	4.8±0.2
	PS1319	3.3±0.2	3.9±0^{b}	4±0.2	4±0.1^{a}	3.8±0.2^{a}	4.4±0.1^{a}	4.2±0.2	4.1±0.2	4.2±0.2
	Sig.	NS	**	NS	**	**	*	NS	NS	*
T°C	PS1319 ST	24.8±0.2^{a}	25.9±0.1^{c}	30.3±0.2^{b}	35.2±0.1^{a}	46.4±0.1^{c}	43.7±0.7^{a}	46±0.3^{b}	47.8±0.1^{b}	na
	PS1319 T	24.4±0.2^{a}	24.8±0.1^{a}	25.8±0.9^{a}	36.6±0.1^{b}	30.7±0.3^{a}	47.4±0.1^{b}	43.2±0.3^{a}	47.7±0.4^{b}	47.2±0.2
	PS1319	25.6±0^{b}	25.3±0.2^{b}	25.1±0.2^{a}	40.7±0.1^{c}	41±0.2^{b}	48.3±0.1^{c}	48±0.1^{c}	46.5±0.3^{a}	47±0.7
	Sig.	***	***	***	***	***	***	***	**	NS
Yeasts, log_{10} CFU/g	PS1319 ST	7±0^{c}	6.5±0^{b}	5.3±0^{b}	5.6±0^{c}	2.5±0.2^{b}	2.4±0.1	na	3.8±0.1^{c}	na
	PS1319 T	6.5±0^{b}	6.6±0^{b}	5.7±0.1^{c}	4.8±0.1^{b}	2.2±0.2^{a}	2.6±0.1	2.4±0.1	2.8±0.1^{b}	5.3±0
	PS1319	5±0^{a}	4.3±0.1^{a}	5±0.1^{a}	4.2±0.2^{a}	5.2±0^{c}	2.4±0.4	na	2.2±0.2^{a}	2.3±0
	Sig.	***	***	***	***	***	NS		***	***
AAB, log_{10} CFU/g	PS1319 ST	4,9±0.5^{a}	5.7±0.2^{a}	5.3±0^{a}	5.6±0^{b}	4±0^{c}	5.5±0^{c}	7±0^{c}	7±0	na
	PS1319 T	6.7±0^{c}	5.5±0^{a}	5.7±0^{b}	5.7±0.1^{b}	2.5±0.5^{a}	2.5±0.1^{b}	4±0^{a}	3±0	2.6±0.1
	PS1319	6±0^{b}	6.6±0^{b}	5.4±0.1^{a}	5.2±0^{a}	3.1±0.1^{b}	2.3±0^{a}	6.2±0^{b}	na	5.6±0.1
	Sig.	***	***	***	***	**	***	***	***	***

B	Fermentation code	Fermentation time (h)								
		0	24	48	72	96	120	144	168	192
pH	SJ02 T	3.5±0.2	3.8±0	4±0.2	4.1±0.1	4±0.2	4.6±0.1	4.6±0.3	4.4±0.2	4.6±0.2
	SJ02	3.5±0.2	4.2±0	3.9±0.2	4.1±0.1	3.9±0.2	4.7±0.1	4.3±0.4	4.6±0.1	4.4±0.3
	Sig.	NS	***	NS	NS	NS	NS	NS	NS	NS
T°C	SJ02 T	24.9±0	24.5±0.1	25±0.2	34.3±0.7	36.1±0.2	46±0.1	45.6±0.3	47.9±0.2	48.2±0.2
	SJ02	25.8±0	24.7±0.2	24.6±0.2	40.2±0.1	35.2±0.8	46.1±0.1	46.1±0.5	47.1±0.3	48.6±0.2
	Sig.	***	NS	NS	***	NS	NS	NS	*	*
Yeasts, log_{10} CFU/g	SJ02 T	6.8±0	6.6±0	6.5±0	3.3±0.1	na	2.9±0.1	2.2±0.2	3.8±0.1	3.4±0.3
	SJ02	4.9±0	5.7±0.1	6.5±0.1	5.2±0	3.6±0.1	2.4±0.1	2.5±0	4.5±0	3.7±0
	Sig.	***	***	NS	***	***	***	NS	***	NS
AAB, log_{10} CFU/g	SJ02 T	5.7±0.1	6.1±0	6.6±0	3±0	3±0.1	2.9±0.1	2.4±0.4	6.7±0.1	6.6±0
	SJ02	5.1±0	6.2±0	5.3±0	5.3±0	3±0	2.4±0.1	na	6.5±0	5.9±0.5
	Sig.	***	***	***	***	NS	**		**	NS

Table 2

Fermentation code	Species	Microbial counts expressed as Log cell/g during fermentation time (h)								
		0	24	48	72	96	120	144	168	192
PS1319 ST	*S. cerevisiae*	5.7±0.2	4.4±0.1	7.4±0	6.7±0.2	5.7±0.1	6.1±0.1	6.2±0	5.7±0	n.a.
PS1319 ST	*T. delbrueckii*	4.7±0.1	5.1±0	5±0.1	4±0.2	3.4±0.2	3.6±0	3±0.1	2.6±0.2	n.a.
PS1319 T	*T. delbrueckii*	4.8±0.1	4.7±0.1	4.7±0.1	6±0.1	4.5±0	4.6±0.2	3.2±0.1	3.5±0.1	4±0
PS1319	*S. cerevisiae*	3.9±0.2	3±0.1	4.3±0.5	5.3±0.2	5.4±0.2	6.4±0.1	5±0.2	4.1±0.3	4.8±0.5
PS1319	*T. delbrueckii*	*	*	*	2±0.3	*	2.6±0.2	*	2.2±0.1	2.5±0.2
SJ02 T	*T. delbrueckii*	4.7±0	5.5±0.2	4.9±0.1	5.1±0.1	4.7±0.1	4.8±0.1	4.2±0.4	3.7±0.1	4.3±0.1
SJ02	*T. delbrueckii*	2.1±0.2	*	*	2±0.4	*	2.3±0.3	*	2.2±0.3	*

Table 3

Concentration (%)

Compound	Group	Odor attribute[1]	Sample code						
			PS1319 ST	PS1319 T	PS1319	Sig.	SJ02 T	SJ02	Sig.
2-phenylacetic acid	Acid	Floral, sweet, honey	6.1±1.3	8.7±0.4	6.6±0.4	NS	10±0.2	8.5±0.2	NS
4-methylvaleric acid	Acid		nd	nd	0.1±0		0.2±0	0.2±0	NS
1-decanoic acid	Acid	Rancid, fatty	0.6±0.1	0.5±0	0.3±0.1	NS	0.2±0.1	0.3±0.1	NS
palmitinic acid	Acid		0.7±0.3	1.3±0.3	0.7±0.1	NS	0.9±0.1	0.8±0.1	NS
hexanoic acid	Acid	Sweet, pungent, rancid	$0.7±0^{b}$	$0.6±0.1^{ab}$	$0.4±0^{a}$	*	0.4±0.2	0.5±0.2	NS
isovaleric acid	Acid	Sweat, acid, rancid	$3.6±0.1^{b}$	$3.2±0.4^{b}$	$2.1±0.3^{a}$	*	4.9±0.2	4.8±0.2	NS
3-hydroxy-2- methyl-4-pyrone (Maltol)	Acid	Malt, roasted-nuts	$0.5±0.1^{a}$	$0,8±0^{b}$	$0,5±0,1^{a}$	*	0,6±0	0,7±0	**
1-nonanoic acid	Acid	Green, fatty	0.4±0.1	0,7±0,4	0,2±0,1	NS	0,4±0,2	0,3±0,2	NS
1-octanoic acid	Acid	Sweet, cheese, oily, fatty	1.6±0.2	1,9±0,2	1,2±0,1	NS	1,9±0,4	2,1±0,4	NS
1-pentanoic acid (valeric acid)	Acid	Putrid, faecal, sweaty, rancid	0.1±0	nd	nd		nd	nd	
propanonic acid	Acid	Pungent, rancid	1.1±0.1	1,3±0,1	1±0,1	NS	1,1±0,1	1,1±0,1	NS
Total			*15.2±1.8^{ab}*	*19±1,2^{b}*	*13,1±0,6^{a}*	*	*20,7±1,2*	*19,3±1,2*	*NS*
1,2-propanediol	Alcohols		1.7±0.4	1,2±0,2	1,4±0	NS	1,1±0	1,3±0	NS
2,3-butanediol	Alcohols	Cocoa butter	$49.2±3^{b}$	$35,4±3,1^{a}$	$61,8±0^{c}$	**	46,3±1,8	47,8±1,8	NS
2-phenylethanol	Alcohols	Rose, honey, fragrant, floral	$7±0.2^{c}$	$3,9±0,4^{b}$	$2±0,1^{a}$	***	3,6±0,6	3,1±0,6	NS
benzyl alcohol	Alcohols	Sweet, flower	$0.2±0^{b}$	$0,2±0^{b}$	$0,1±0^{a}$	*	0,2±0	0,2±0	NS
furfuryl alcohol	Alcohols	Cooked-sugar	$0.7±0^{a}$	$0,9±0,1^{b}$	$0,6±0,1^{a}$	*	0,7±0,1	0,9±0,1	NS
Total			*58.7±3.2^{b}*	*41,6±2,8^{a}*	*65,9±0^{b}*	**	*51,9±1,2*	*53,4±1,2*	*NS*
2-acetylpyrrole	Aldehydes and ketones	Chocolate, hazelnut	$2.7±0.2^{a}$	$3,9±0,3^{b}$	$2,3±0,1^{a}$	*	2,8±0,7	3,6±0,7	NS
2-phenyl-2-butenal	Aldehydes and ketones	flowery, cocoa, sweet, roasted	$0.3±0^{c}$	$0,2±0^{b}$	$0,1±0^{a}$	**	0,1±0	0,2±0	NS
2-pyrrolaldehyde	Aldehydes and ketones		$0.3±0^{b}$	$0,3±0^{b}$	$0,2±0^{a}$	*	0,3±0,1	0,4±0,1	NS
2-pyrrolidinone	Aldehydes and ketones		$0.7±0.1^{a}$	$1,5±0^{b}$	$1,5±0,1^{b}$	**	1,7±0,1	2±0,1	NS
acetoin	Aldehydes and ketones	Butter cream	$3.4±0.4^{a}$	$9,4±0,1^{b}$	$3,8±0,9^{a}$	**	5,6±1,5	5,2±1,5	NS
benzaldehyde	Aldehydes and ketones	Bitter almonds, grass	0.6±0.1	0,7±0,2	0,4±0,1	NS	0,7±0,1	0,7±0,1	NS
furfural	Aldehydes and ketones	Almonds	$0.1±0^{a}$	$0,2±0^{b}$	$0,1±0^{a}$	*	0,2±0,1	0,1±0,1	NS
Total			*8.1±0.2^{a}*	*16,1±0,7^{b}*	*8,3±0,7^{a}*	***	*11,5±1*	*12,2±1*	*NS*

eEthyl oleate (oleic acid ethyl ester)	Esters		0.1±0	0,2±0	0,1±0,1	NS	0,2±0	0,1±0	NS
phenethyl acetate	Esters	Rose, honey, flowery	4.6±0.1[b]	3,8±0,5[a]	1,7±0,1[a]	**	2,6±0,4	2,1±0,4	NS
ethyl dodecanoate	Esters	Fruity, floral	1.2±0.1[c]	1±0[b]	0,6±0[a]	**	0,6±0,1	0,6±0,1	NS
ethyl linoleate (òinoleic acid ethyl ester)	Esters		0.3±0.1	0,6±0,1	0,3±0,1	NS	0,4±0	0,3±0	NS
ethyl myristate (Myristic acid ethyl ester)	Esters		0.8±0.1[b]	0,9±0[b]	0,5±0,1[a]	*	0,5±0	0,4±0	NS
ethylphenyl acetate	Esters	Fruit, sweet, honey	0.2±0	nd	nd		nd	nd	
methyl palmitate (palmitic acid, methyl ester)	Esters		0.3±0	0,7±0,1	0,6±0,3	NS	0,4±0	0,3±0	NS
Total			*7.5±0.2[b]*	*7,3±0,7[b]*	*3,8±0,4[a]*	**	*4,7±0,6*	*3,8±0,6*	*NS*
2,3-dihydro-benzofuran	Furans		0.6±0.1[a]	1,8±0[c]	1,4±0,2[b]	**	0,7±0,3	1±0,3	NS
Total			*0.6±0.1[a]*	*1,8±0[c]*	*1,4±0,2[b]*	**	*0,7±0,3*	*1±0,3*	*NS*
gamma-butyrolactone	Lactones	Smoked	0.8±0[a]	1,7±0,1[b]	1,1±0,1[a]	**	1,3±0,2	1,6±0,2	NS
gamma-crotonolactone	Lactones		0.3±0	0,4±0,1	0,3±0	NS	0,3±0	0,4±0	*
solerone	Lactones	Fruity	1.8±0.2	0,7±0	nd	*	±	±	
Total			*2.9±0.2[b]*	*2,7±0,2[b]*	*1,3±0,1[a]*	**	*1,6±0,2*	*2±0,2*	*NS*
2,3-dihydro-3,5-dihydroxy-6-methyl-4H-Pyran-4-one	Pyrans		6.8±1.3[a]	11,1±0[b]	5,8±0,2[a]	*	8,2±0,2	7,3±0,2	*
Total			6.8±1.3[a]	11,1±0[b]	5,8±0,2[a]	*	8,2±0,2	7,3±0,2	*
2,3-Benzopyrrole (indole)	Pyrrols		0.3±0	0,4±0	0,4±0,1	NS	0,7±0,2	0,9±0,2	NS
Total			*0.3±0*	*0,4±0*	*0,4±0,1*	*NS*	*0,7±0,2*	*0,9±0,2*	*NS*