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Running title: Development of bacteria and yeast in cocoa fermentation

ABSTRACT

Forastero hybrid cocoa bean fermentations were carried out in Box (B) and in Heap (H) with or without the inoculation of *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* as starter cultures. Bacteria, yeasts and microbial metabolites (volatile and non-volatile organic compounds) were monitored during fermentation in order to assess the link between microbiota and the release of metabolites during this process. The presence of starter cultures was detected during the first two days of both fermentations by means of culture-dependent analysis. However, it did not show statistical difference in any physico-chemical or microbiological analysis. Plate counts revealed the dominance of yeasts at the beginning of both fermentations followed by acetic acid bacteria (AAB) and lactic acid bacteria (LAB). *Hanseniaspora opuntiae, S. cerevisiae, Pichia pijperi, Acetobacter pasteurianus* and *Lactobacillus fermentum* were the most abundant OTUs during both fermentation processes (B and H), reporting different relative abundances. Only the diversity of fungal species indicated a higher level of complexity in B compared to H fermentations ($P < 0.05$) and also revealed a statistically significant difference between starter cultures initially inoculated ($P < 0.01$). However, the analysis of microbial metabolites indicated different distribution of volatile and non-volatile compounds between the two procedures B and H ($P < 0.05$), rather than between the inoculated and non-inoculated fermentations. Box fermentations showed a faster carbohydrate metabolism and higher production of organic acid compounds than in heap fermentations, which boosted the formation of alcohols and esters. Overall, the microbial dynamics and associations between bacteria, yeast and metabolites were found to depend on the type of fermentation.

IMPORTANCE

In spite of the limited effectiveness of the starter strains inoculated, this study provides new information on the microbial development of Box and Heap cocoa fermentations, under inoculated and non-inoculated conditions, by coupling yeast/bacteria amplicon-based sequencing data with microbial metabolites detection. The information so far

available suggests that microbial communities have been an important factor in the evolution of aroma compounds. Understanding the pathways taken place during the formation of aroma by micro-organisms could be used to improve fermentation processes and to enhance chocolate quality.

KEYWORDS Cocoa beans; fermentation; yeast; bacteria; volatile organic compounds; non-volatile organic compounds; HTS

INTRODUCTION

Cocoa (*Theobroma cacao* L.) is an important plant crop worldwide and its production serves as a major source of income in several developing countries (1). According to the Food and Agriculture Organization (FAO) in 2016, the world cocoa bean production was 4,466,574 tonnes (2). In terms of overall amount of beans per country, in 2016 the major cocoa-producing countries were Ivory Coast followed by Ghana, Indonesia, and Cameroon (2). The chocolate production begins by harvesting the cocoa fruit, where cocoa beans and the surrounded mucilaginous pulp inside the pods are removed. At this point, the product has an astringent characteristic and needs to be fermented, dried, and roasted in order to acquire the optimal features of cocoa flavor and taste (3). Normally, spontaneous fermentation goes on from 3 to 10 days in heap, box, basket or tray.

According to Schwan and Fleet (4), the microbiota present during cocoa fermentation is composed by yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB). Two important stages occurred during cocoa fermentation; in the first stage, yeasts proliferate reducing sugars and citric acid from the pulp and producing ethanol and carbon dioxide. Alongside, temperatures and pH increase due to aerobic and oxidative reactions, allowing the growth of LAB and AAB (4). LAB transforms sugars and organic acids mainly into lactic acid and, under aerobic conditions, AAB converts ethanol to acetic acid (3). The second stage involves the death of the seed embryo due to the high concentrations of ethanol and acetic acid, and the increase in temperatures (3). The quality of the end product chocolate depend on the three groups of microorganisms cited above, since they are able to produce metabolites and flavor precursors (4).

Despite, the importance of yeasts during cocoa fermentation showed in recent studies (5, 6), fungal biodiversity in fermented food has been studied far less than bacteria. In spite of the application of high-throughput sequencing (HTS) this decade, this new technology has been largely used to give new insights in the domain of fermented foods by enable to discover, validate and screen genetic variants of a complex ecosystem (7). The importance of

identifying the microbial composition in food ecosystems is to find appropriate starter cultures that enhance a particular aspect of the product. Recently, *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* have been detected and used as starter culture in cocoa fermentation, showing a positive impact on the aroma profile of the end product (8, 9). However, there has been much controversy concerning the choice of starter cultures used in cocoa fermentation to improve the quality of the end product. This paper involves the importance of the reproducibility of two starter cultures already used in cocoa fermentations under changing conditions. We highlight the fact that reproducibility depends on the initial microbial population and their importance on aroma development.

The present study aimed to determine the dynamics and biodiversity of both bacteria and yeasts by amplicon based sequencing of the 16S rRNA genes and the ITS2 gene, respectively, during cocoa beans fermentation carried out spontaneously and in presence of yeasts starter cultures, either in boxes or heaps to acquire a deeper knowledge about the relationship of microorganisms between each other and their surroundings. Analyses of non-volatile and volatile organic compounds were also assessed with the aim to investigate how the use of cultures can affect the volatilome profile of fermented cocoa under the two different fermentation processes. In this study, we also proposed the measurements of associations between microbial communities and the development of microbial volatile and non-volatile compounds. Undoubtedly, a better understanding of the microbial communities and physico-chemical dynamics during box and heap fermentations will help to develop new management procedures for the production of high-quality cocoa.

RESULTS

Physical and microbiological changes throughout box and heap fermentations. Temperature and pH were measured during Box and Heap (B and H) fermentations at time 0 and after 48, 96 and 120 h as shown in Table 1. No significant difference ($P > 0.05$) between the conditions used (inoculated and non-inoculated) was observed through physical and

microbiological analysis, while the temperature observed during B and H fermentations significantly increased from initial values of 27 °C to 43 °C and 40 °C, respectively at the end of the fermentation ($P < 0.05$). Regarding the pH of the cocoa bean-pulp, at the beginning of the trial was 3.5, and increased to 4.2 and 4.7 at the end of the fermentation for B and H fermentations respectively ($P < 0.05$).

Yeasts, LAB and AAB population dynamics are reported in Table 1. Yeasts were the dominant population until 48 hours in both processes (B and H), and they were detected at high loads in the cocoa beans already before the inoculum of the starter strains, with an average value of 6.98 log CFU g^{-1} in H and 7.14 log CFU g^{-1} in B fermentations. Yeast population reached the highest load at 48 hours and decreased rapidly below the limit of detection of microbiological analysis ($P > 0.01$) after that sampling point. In contrast, in H fermentation the yeast population maintained loads at around 7 log CFU g^{-1} also after 48 hours, with the higher counts recorded at the end of the process (7.57 log CFU g^{-1}). A significant difference between B and H was also observed in LAB dynamics throughout fermentation time, with a marked increase of the counts after 48 hours in both fermentation processes (B: 5.91 to 6.55 and H: 5.78 to 7.76 log CFU g^{-1}), as shown in Table 1 ($P < 0.01$). High counts of AAB were observed at the beginning of B and H fermentations (6.32 and 6.17 log CFU g^{-1}, respectively). However, this population showed a fluctuation behavior throughout B fermentation time, whereas, during H fermentation increased over time to final counts of 8.00 log CFU g^{-1} ($P < 0.01$). It is noteworthy that, after 96 hours AAB dominated over LAB and yeasts in both fermentation processes. Overall, at 96 hours we observed higher counts for the three microbial groups considered (yeasts, LAB, AAB) in H fermentation compared to B as shown in Table 1 ($P < 0.05$).

Identification of isolated yeast colonies and assessment of starter strains dominance. In relation to the observed yeast dynamics in B and H fermentations, one hundred and four yeast colonies were isolated from WL Nutrient agar plates. The ITS-RFLP fingerprints identified *S. cerevisiae* and *T. delbrueckii* in 70 % of the isolated colonies.

Furthermore, REP-PCR fingerprints and the comparison with starter profiles highlighted the presence of *S. cerevisiae* ID76 and *T. delbrueckii* ID103 in the cultivable mycobiota during the first 48 hours of both B and H fermentations. *S. cerevisiae* ID76 represented 68 % of the isolates from fermentations inoculated with *S. cerevisiae* (S) and 51 % of colonies isolated from fermentations inoculated with *S. cerevisiae* and *T. delbrueckii* (ST). Lastly 38 % of the colonies isolated from fermentations ST were ascribed to *T. delbrueckii* ID103 profile. Besides the identification of the starter strains, *Hanseniaspora opuntiae* represented the most abundant autochthonous species, representing 31 % of the colonies isolated from non-inoculated fermentations (data not shown).

Dynamics of non-volatile organic compounds during cocoa beans fermentation. The evolution of non-volatile compounds were determined during B and H fermentations of cocoa beans by High-Performance Liquid Chromatography (HPLC) as shown in Figure 1. No significant differences between inoculated and non-inoculated fermentations were observed through non-volatile compounds analysis. At the beginning of the process, B fermentations showed higher concentrations of glucose, fructose and sucrose (24.00, 24.93 and 8.13 mg/g, respectively) compared to H fermentations (19.53, 22.88 and 9.90 mg/g, respectively) and a significantly decreased of glucose, fructose and sucrose during both fermentation processes (B and H) was observed over fermentation time ($P < 0.05$).

Regarding the overall content of organic acids, in the cocoa bean-pulp before the start of the fermentation, citric acid showed the highest concentration, followed by succinic, and gluconic acid in both fermentation processes (Fig. 1). Noteworthy, higher amounts of lactic and succinic acids were detected at 48 hours, whereas the maximum production of acetic acid was observed at 96 hours. From 48 hours to the end of both fermentation processes (B and H), succinic acid represented the most abundant organic acid, with concentrations up to 21.37 and 18.07 mg/g, respectively. The dynamics across time observed for organic acids during both fermentation processes (B and H) were similar. A statistically significant decrease of citric and gluconic acid concentrations were observed along B and H fermentations, reaching the

lowest values at the end ($P < 0.01$). In contrast, an increase of malic, succinic, lactic and acetic acid concentrations during fermentation time ($P < 0.01$) was found in both processes (B and H). No significant changes were observed for oxalic, pyruvic, tartaric and fumaric acids during B or H over fermentation time (see Table S1).

Volatilome during cocoa bean-pulp fermentation. A total of 72 volatile organic compounds (VOCs) were identified by Head-Space Solid Phase Micro-Extraction Gas Chromatography-qMass Spectrometry (HS-SPME /GC-qMS) on fermented cocoa bean-pulp (see Table S1). No significant differences between inoculated and non-inoculated fermentations were observed through VOCs analysis. At the beginning of B and H fermentation processes 2-pentanol, ethyl acetate, limonene and 1,2-propanediol diacetate were the most abundant volatile compounds found, whereas at the end of both fermentations acetic acid, limonene, 2-heptanol, phenylethyl alcohol, isopentyl alcohol, isovaleric acid and benzeneacetaldehyde represented the most retrieved VOCs in the headspace (see Table S1). Noteworthy, a significantly higher concentrations of total VOCs was found at the end of B fermentation (21,859.59 µg/kg) compared to H (11,208.63 µg/kg) (see Table S1, $P < 0.01$).

Mycobiota of cocoa beans during fermentation. A total of 1,304,936 raw reads (2x250 bp) were obtained and 1,217,061 reads passed the filters applied through QIIME, with an average value of 31,975 ± 22,635 reads/sample, and a mean sequence length of 411 bp. The rarefaction analysis and the estimated sample coverage were satisfactory for all samples, with an ESC average of 97 % (see Table S2), and the alpha-diversity indicated a higher level of complexity in B compared to H fermentations ($P < 0.05$). Overall, 18 fungal OTUs were identified during the fermentations as shown in Table 2. A statistically significant difference between conditions was found, with a higher relative abundance of *Hanseniaspora opuntiae* in non-inoculated fermentation (46.23 %) compared to those inoculated with with *S. cerevisiae* and *T. delbrueckii* (ST) (25.60 %, $P < 0.05$). In addition, significantly higher

195 presence of *T. delbrueckii* in fermentations inoculated with the mixed yeasts culture (ST,
196 22.23 %) compared with the inoculated only with *S. cerevisiae* (S) and the non-inoculated
197 (0.03 and 0.11 %, respectively) was observed ($P < 0.01$, Table 2).

198 Inoculated cocoa beans (S and ST) in both fermentations processes (B and H) at time
199 0 showed the dominance of *H. opuntiae, Candida jaroonii, S. cerevisiae, T. delbrueckii* and
200 *Pichia pijperi* (Table 2). In addition, *H. opuntiae, P. pijperi,* and *C. jaroonii* were the most
201 predominant in non-inoculated B fermentations at the beginning of the process, while in non-
202 inoculated H fermentations, *H. opuntiae, P. pijperi,* and *Botryosphaeria* reached the highest
203 incidence. However, at the end of both fermentation methods (B and H) *H. opuntiae*, *S.*
204 *cerevisiae, P. pijperi* and *Kluyveromyces marxianus* were the most abundant. Mycobiota
205 dynamics were similar over time during inoculated and non-inoculated in B and H
206 fermentations. *S. cerevisiae* significantly increased across time in both processes, while *H.*
207 *opuntiae* significantly decreased as shown in Table 2 ($P < 0.01$).
208
209 **Bacterial community of fermented cocoa beans.** The total number of paired
210 sequences obtained from fermented cocoa beans reached 4,159,213 raw reads. After merging,
211 a total of 2,655,230 reads passed the filters applied through QIIME, with an average value of
212 63,220 ± 45,781 reads/sample, and a mean sequence length of 445 bp. The rarefaction
213 analysis and Good's coverage, expressed as a percentage (91 %), indicated also satisfactory
214 coverage for all samples (see Table S3). Alpha-diversity indicated a higher level of
215 complexity only across fermentation time (see Table S3, $P < 0.05$). No significant difference
216 was observed when comparing the different conditions (inoculated with S or ST and non-
217 inoculated) or between processes (B and H).

218 Overall, in both inoculated and non-inoculated B fermentations the most abundant
219 OTUs detected at 48 hours were *Acetobacter pasteurianus, Lactobacillus fermentum* and *L.*
220 *plantarum* (Fig. 3). Notably, *A. pasteurianus* and *L. fermentum* remained the two most
221 abundant OTUs at the end of the box fermentation at all conditions (inoculated or non-

inoculated), followed by *Bacillus*. Regarding the inoculated H fermentations, *A. pasteurianus*, *L. fermentum* and *Acetobacteraceae* were the most abundant OTUs detected at 48 hours and along the entire fermentation time *A. pasteurianus* and *L. fermentum* remained the dominant OTUs (Table 3). In contrast, non-inoculated H fermentation were characterized by a high relative abundance of *L. fermentum*, *A. pasteurianus*, and *L. plantarum* at 48 hours while, at the end of the process, *L. fermentum*, *Bacillus* and *Klebsiella* took over and dominated. As far as the dynamics are concerned under the different conditions, we observed an increase of relative abundances for *L. fermentum, L. plantarum, A. pasteurianus, Bacillus*, *Acetobacteraceae* and *Lactobacillaceae* along fermentation time, while *Erwinia*, *Gluconobacter, Trabulsiella* and *Enterobacteriaceaee* decreased during time ($P < 0.01$) as shown in Table 3.

OTUs co-occurrence and/or co-exclusion during cocoa bean fermentation. When plotting the relative abundance of bacterial and yeast populations, considering OTUs of all conditions together (inoculated with S, ST and non-inoculated) of each fermentation method (B and H), it was possible to observe microbial co-occurrence or co-exclusion dynamics between the two different communities as shown in Figure 2.

Overall, in B fermentations (Fig. 2A) *L. plantarum, A. pasteurianus* and *Enterobacteriaceaee* were negatively associated with the main yeast OTUs (*S. cerevisiae, K. marxianus, C. inconspicua* and *P. pijperi).* In detail, *S. cerevisiae* was positively correlated with *Acetobacteraceae* and *Lactobacillaceae*, whereas *A. pasteurianus* was positively correlated with *K. marxianus* and *C. inconspicua* and negatively correlated with *C. jaronii* and *H. opuntiae* ($P < 0.05$). However, *H. opuntiae* was positively associated with the presence of the *Enterobacteriaceaee* family as well as with *Gluconobacter* ($P < 0.05$). Noteworthy, *H. opuntiae* and *C. jaronii* were found positively associated with the minor OTUs, *Citrobacter* and *Erwinia* ($P < 0.05$, Figure 2A).

In H fermentations, *L. fermentum* showed a positive correlation with *K. marxianus* and *C. inconspicua* and a negative correlation with *C. jaronii* ($P < 0.05$) as observed in Figure

2B. *S. cerevisiae* was positively correlated with the *Acetobacteraceae* and with *A. pasteurianus (*Figure 2B).

Correlation between sugar and organic acid compounds and microbiota populations detected by HPLC. Significantly different correlations pattern between sugars, organic acids and microbes, in B and H fermentations were observed, as shown in Figure 3 ($P < 0.05$). Overall, in B fermentations the most abundant microbial species in fermented cocoa beans such as, *H. opuntiae, A. pasteurianus, K. marxianus, L. plantarum* and *S. cerevisiae* were statistically positively correlated with sucrose, glucose, citric acid, succinic acid and lactic acid ($P < 0.05$), while the *Bacillus, S. cerevisiae, L. plantarum, A. pasteurianus* and *L. fermentum* were statistically negatively correlated with sucrose, fructose, glucose, citric acid, succinic acid, gluconic acid and pyruvic acid ($P < 0.05$). In addition, sucrose was positively correlated to the presence of *H. opuntiae* ($P < 0.05$) and negatively with *A. pasteurianus* and *Bacillus* ($P < 0.05$). Citric acid was negatively correlated with *Bacillus* and *S. cerevisiae,* but positively with *H. opuntiae, Gluconobacter* and *Erwinia* ($P < 0.05$). *L. fermentum* was negatively correlated to fructose, glucose, gluconic acid and pyruvic acid ($P < 0.05$). Finally, succinic acid was positively associated with *A. pasteurianus, C. inconspicua* and *K. marxianus* and lactic acid positively related to *L. plantarum* and *S. cerevisiae* as shown Figure 3A ($P < 0.05$).

In contrast few statistically significant correlations were found in H fermentations (Figure 3B) were *A. pasteurianus, K. marxianus, L. plantarum, C jaroonii* and *L. fermentum* were positively correlated with sucrose, fructose, glucose, citric, pyruvic acid, succinic and lactic acid. In detail, *A. pasteurianus* was found to be positively associated with sucrose, while *Gluconobacter* was negatively related with sucrose ($P < 0.05$). In addition, *A. pasteurianus, K. marxianus, L. plantarum,* and *L. fermentum* were positively associated with succinic and lactic acid as shown in Figure 3B ($P < 0.05$).

Correlation between microbiota and volatilome profile. Significantly different associations between secondary metabolites and the main OTUs were observed in B and H fermentations, as showed in Figure 3 ($P < 0.05$). In B fermentations (Fig. 3A), the major bacterial and fungal taxa such as, *S. cerevisiae, H. opuntiae, L. plantarum, A. pasteurianus, K. marxianus, C. inconspicua* and *L. fermentum* were statistically correlated with key-aroma and fermentative markers, while the minor OTUs bacteria (*Enterobacteriaceaeee, Trabulsiella, Erwinia,* and *Gluconobacter)* and *H. opuntiae* were statistically negatively correlated with acids and phenols. In detail, positive correlations were found between *S. cerevisiae* with ethyl octanoate, 2-methyl-butanal, 3-methyl-butanol, *H. opuntiae* with 2-pentanol ($P < 0.05$), *L. plantarum* with 2-heptanol, 2-methyl-butanal, 3-methyl-1-butanol and ethanol, and *L. fermentum* with ethyl octanoate, 2-heptanol, benzyl alcohol and isovaleric acid ($P < 0.05$). In addition, *A. pasteurianus*, *C. inconspicua* and *Bacillus* were also positively correlated with acetoin, acetic acid, isovaleric acid, phenol, limonene, benzyl alcohol and phenylethyl alcohol ($P < 0.05$), while these compounds were positively correlated with *H. opuntiae* and the minor bacterial OTUs as shown in Fig 3A ($P < 0.05$).

In H fermentations less correlations were observed compared with B fermentation (Fig. 3B). In general, some of the most abundant microbes (*A. pasteurianus, T. delbrueckii, S. cerevisiae, K. marxianus)* and *Acetobacteraceae* showed several significantly positive correlations with VOCs. *A. pasteurianus* was positively correlated with ethyl octanoate, 2-heptanol, 2-hepanone, *cis*-furan-linalool oxide, benzaldehyde, acetoin, β-phenylethylacetate, 3-methyl-1-butanol, limonene, 2-pentanol acetate, phenylethyl alcohol, ethanol and isopentyl alcohol ($P < 0.05$). *T. delbrueckii* was positively associated with 2-heptanone ($P < 0.05$). *S. cerevisiae* was positively correlated with 3-methyl-1-butanol and ethanol ($P < 0.05$). Finally, *Acetobacteraceae* was positively correlated to ethyl octanoate, 2-heptanol, 2-heptanone, *cis*-furan-linalool oxide, 3-methyl-1-butanol, acetoin, limonene, phenylethyl alcohol, ethanol and isopentyl alcohol, while *K. marxianus* was positively correlated to benzaldehyde, acetoin, acetic acid, benzyl alcohol and β-phenylethylacetate ($P < 0.05$).

DISCUSSION

In this study, the changes in physico-chemical composition, microbial counts and microbiota diversity in two different fermentation processes, box (B) and heap (H), inoculated or not with yeasts as a starter culture, were investigated. The ability of the survival and grow of selected starter strains in this case *S. cerevisiae* ID67 and *T. delbrueckii* ID103 during cocoa fermentation is one of the most important feature to ensure their effect during this process. These starters showed the ability to coexist with autochthonous microbial communities in fermented cocoa beans. However, the yeast cultures used did not modify significantly the microbiological dynamics, physic-chemical parameters and metabolites produced during fermentation, whereas the same starters influenced the fermentative process and the quality of end-products in at least one cocoa hybrid variety (9). It is important to note that in the previous study, the initial yeast load was lower than those observed in our study and might explained the discrepancies on the impact of the same yeast culture during cocoa fermentation. The influence of fermentation practices, variety of cocoa and the use of different starter cultures on site during the cocoa bean fermentation plays an important role in the success of the starter culture used during fermentation and might explain the discrepancies found between studies (5, 6, 8–14). Our results proof that the effectiveness of the cultures lies on the micro-environments influenced by the geographical sites.

During fermentation, cocoa beans constitute an ecological niche for a wide range of microbes. The advances in studying the dynamics of cocoa microbial communities have shown that the composition of these communities follows predictable patterns reporting a rapid decline in yeast counts after 48 hours when the sugars are depleted, the temperature raise and LAB and AAB increase (15–19). A great impact on the microbial dynamics and succession during cocoa fermentation have been explained by the use of different cultivar varieties, fermentation methods, environmental conditions, harvesting and post-harvesting methods, as well as externals factors such as cross-contamination (equipment, operators, insect interactions and microbial populations from previous fermentations) (14, 16).

The use of molecular biology tools and the improvement of culturing techniques have been facilitating the detection of new species of yeasts, LAB, and AAB. It was already reported that the main microbial communities from fermented cocoa beans has a restricted microbial population, including *H. opuntiae, A. pasteurianus* and *L. fermentum* also detected in our study (8, 13, 20). By the application of amplicon based sequencing we were able to detect unusual yeasts such as *C. jaroonii*, *Lasiodoplia theobromae* and *Botryosphaera* during cocoa fermentation, not previously detected. Despite this restricted community, there are some discrepancies among the dominating microbial species, which may vary considerably within countries and according to region, while there is no information regarding the incidence of minor microbial groups (21).

To gain more knowledge in the range of potential interactions between microbial communities this study describes a possible co-occurrence and co-exclusion. Our results obtained from statistical correlations of bacterial ecology especially LAB showed a modulation by the yeast culture, which matched with the mutualistic and synergetic interactions reported previously for LAB and yeasts (22). Noteworthy, in our study these associations were depending by the type of fermentation process and the correlation dataset was used to explore the drivers of possible microbial dynamics, interactions and metabolism. This information can give an idea about the kinetics of substrate consumption and aroma production by the microbiota present in fermented cocoa beans.

The dynamics of non-volatile compounds showed a successful competition for nutrients by microbial populations within cocoa fermentations. The ability to reduced sugars observed from fungal and bacterial communities from our results has been deeply studied and supported by previous studies (23, 24). Regarding organic acids dynamics, citric acid had the highest concentration at the beginning of both fermentation and decreased overtime. This utilization of citrate has been attribute to bacteria, which metabolized it into acetic acid, carbon dioxide and lactic acid (25). However, not only bacteria can utilize citrate as energy source, some isolates within *C. krusei* have been reported to assimilate citrate during cocoa fermentation (26). However, this specific yeast was not detected in our study during cocoa

fermentation, while the presence of the most abundant yeasts found in this study *H. opuntiae* and *S. cerevisiae* have never showed the capability to assimilated citrate *in vitro* (27). Therefore is hypothesized that citrate assimilation was due to LAB, such as the highly abundant *L. fermentum*, as also supported from previous study (28). The high concentrations of succinic acid at 48 hours to the end of the fermentation is likely related to the LAB metabolic activity, since these bacteria have been shown the capability to produce succinic acid from the citrate fermentation or convert fumaric and malic acids to succinic acid (29, 30). The reduction of pH in the pulp caused by LAB producing lactic acid favors the growth of AAB species, such as *A. pasteurianus* which is capable to produce acetic and malic acids (29, 31).

Biochemical reactions plays a key role in the formation of VOCs in fermented cocoa beans (22, 32). In our study, we observed that the dynamics of VOCs during fermentation changed in the concentration and composition. According to Kone *et al.,* (33) *P. kudriavzevii* and *S. cerevisiae* were the most important producers and contributors of cocoa aroma compounds followed by *Wickerhamomyces anomalus, Geotrichum* and *Pichia galeiformis*. In our study, desirable cocoa aroma compounds such as 2-heptanol, ethyl acetate and, 2-phenylethanol were found in both fermentation processes as previously identified by Ramos *et al.,* (6). The principal producers of alcohol, ester, and acid compounds have been linked to yeasts such as *S. cerevisiae*, *Candida* and other yeast species not found in this study in fermented cocoa beans (33–35). Besides the production of VOCs by fungi, AAB are well known to oxidized alcohols such as ethanol, isoamyl alcohol, 2-phenylethanol to produce acids and acetaldehydes (36, 37).

We observed that the major bacterial group found in our study increased the concentration of succinic, acetic, lactic acids, acetoin, alcohols, esters and acetaldehydes. Overall, the biochemical contribution in food ecosystems might change by the complexity of the microbial consortia (38). Therefore, further research is needed to understand the role of other compounds such as free amino acids, oligopeptides, and polyphenols in the development of microbes and aroma compounds (3, 22).

CONCLUSION

Overall, the polyphasic approach applied in this study allowed us to get new insights into the microbial development and aroma formation during cocoa fermentation. Here, we observed that the starter culture modulated the microbiota composition of fermented cocoa beans and marginally affect the metabolites, which were most influenced by the type of process carried out. Accordingly, the difference found between Box and Heap fermentations might be explained by the environmental and processing conditions, in which the microenvironment of each process play an important role. The application of the omics approach confirmed that fermented cocoa beans have complex microbial communities dominated by a restricted bacterial and yeast populations. Future research is needed to assess how fermentation methods or the presence of starter culture can affect the final characteristics of the chocolate.

MATERIALS AND METHODS

Cocoa bean fermentations. The lyophilized strains *S. cerevisiae* ID67 and *T. delbrueckii* ID103 were provided by Lallemand (Canada, Quebec, Montreal), and were used as starter cultures in farmer-scale cocoa bean fermentations carried out in Ngoumou (Yaoundé, Cameroon) at the end of the middle-crop of 2016 (September-October 2016). The strains were chosen according to the study of Visintin *et al.*, (9). Briefly, cocoa pods of *Forastero* hybrid were harvested by traditional methods and stored on the ground for 2-3 days before the opening of the pods. The cocoa pods were cut with unclean machetes and beans and adhering pulp were taken manually by hand. Approximately 3 h after breaking the pods, cocoa beans-pulp were grouped in two independent processes (Box and Heap). Approximately, 200 kg of fresh cocoa beans-pulp were used for B fermentation, placed in wooden box (0.06 m^3), covered with banana leaves and closed with a wooden lid to avoid open air. The heap fermentations were set up with smaller amount of beans compare to the box fermentation due to the fact that an adult can manually turn no more than 100 kg of beans-pulp, these beans were piled on banana leaves and covered with the same leaves and

414 jute rags. The field experiment was settled inoculating the cocoa beans-pulp with *S.*
415 *cerevisiae* ID67 (S) or with *S. cerevisiae* ID67 in co-culture with *T. delbrueckii* ID103 (ST) in
416 a ratio 1:1 (weight: volume) at the beginning of both fermentation processes (B and H). The
417 lyophilized starter cultures were revitalized in sterile saline solution for 30 min at room
418 temperature and were progressively added and mixed to the cocoa-pulp mass at final
419 concentrations of 7.0 ± 0.2 Log CFU g^{-1}. In addition, non-inoculated fermentations were
420 carried out as a control, without adding any starter culture in both fermentation processes (B
421 and H). All trials were performed in duplicate (n=12) from an on-site fermentation, following
422 local agricultural practices: cocoa bean-pulp mass was turned manually at 48 and 96 h and
423 after 120 h the fermentations were stopped by spreading the beans on a drying platform. For
424 each of the six experimental trials, 1-1.5 kg of cocoa-pulp was collected in sterile bags after 0,
425 48, 96, and 120 h, randomly from at least five random zones of the fermentative mass of each
426 B or H fermentations. Noteworthy, sampling at 48 and 96 h were performed before mixing
427 the mass. From the collected beans, approximately 20 g of samples were collected, stored at -
428 20°C and transported on dry ice to the Department of Agriculture, Forestry and Food Sciences
429 (University of Turin, Italy) for further metabolites analysis. Aliquots of 25 g of each sample
430 underwent microbiological analyses at the experimental laboratory set up on site. The pH
431 values and temperatures were measured at same sampling time during fermentation by an
432 average of five random zones of the cocoa bean-pulp mass, by using a pH-thermometer
433 (Crison, Modena, Italy).
434
435 **Culture-dependent microbial community dynamics.** Classical microbiological
436 analysis was performed for samples recovered at 0, 48, 96 and 120 h. Twenty-five grams of
437 cocoa beans and adhering pulp were homogenized with 225 mL of Ringer's solution (Oxoid,
438 Milan, Italy). Decimal dilutions in quarter-strength Ringer's solution were prepared. Aliquots
439 of 0.1 ml of the appropriate dilutions were spread in triplicate on the following media: WL
440 Nutrient agar (WLN; Lab M, Heywood, Lancashire, UK) plus 1 µg/mL of tetracycline
441 (Sigma-Aldrich, Milan, Italy) for counting total yeast incubated for 3/5 days at 30°C, De Man

442 Rogosa and Sharpe agar (MRS, Oxoid, Milan, Italy) plus 2 µg/mL of natamycin (Sigma-
443 Aldrich, Milan, Italy) for growing LAB, incubated at 30°C for 48 h and Acetic Acid Medium
444 (1 % glucose, 0.8 % yeast extract, 0.5 % bacteriological peptone, 15 g/L agar, 0.5 % ethanol,
445 0.3 % acetic acid), plus 2 µg/mL of natamycin (Sigma-Aldrich, Milan, Italy) for growing
446 acetic acid bacteria (AAB) incubated at 30 °C for 3/5 days. Results were expressed as means
447 of Log CFU g^{-1} from three independent determinations. Yeasts colonies (5-8 for each
448 sampling points) were randomly isolated from the highest dilution plate of WLN. These
449 colonies were further purified by streaking and stored in 20 % v/v glycerol. At each sampling
450 point, 1 ml of the first 10-fold serial dilution was collected and directly centrifuged at
451 maximum speed for 30 s.
452
453 **Assessment of yeast ecology by culture-dependent analysis.** DNA extraction from
454 single isolates was performed as described by Cocolin *et al.,* (39), and normalized at 100 ng
455 L^{-1}. Isolates were grouped in relation to their Restriction Fragment Length Polymorphism
456 (RFLP) profiles, which were obtained after enzymatic restriction of the amplified ITS-5.8S
457 rDNA region, as previously described by Korabečná et al., (2003). ITS-5.8S rDNA region of
458 at least three representative isolates of each RFLP-group was for sequencing (GATC Biotech,
459 Colonia, Germany). REP-PCR assay was performed on all isolates previously identified as *S.*
460 *cerevisiae* and *T. delbrueckii* following previous study by Visintin *et al.,* (2017) (9). The
461 presence of starter culture from the REP-PCR profiles was compared with those of *S.*
462 *cerevisiae* ID67 and *T. delbrueckii* ID103.
463
464 **Chemical analysis.** For sugar and organic acid analyses from fermented cocoa bean-
465 pulp under different conditions were extracted by crushing the cotyledon and pulp using a
466 pestle and lyophilized for further analysis. Fermented lyophilized beans-pulp samples (0.20 g)
467 were washed with 2 ml of pure hexane (Sigma-Aldrich, Milan, Italy) and vortexed for 5 min.
468 The homogenate was centrifuged (6000 x g, 4°C for 15 min) and the supernatant was
469 removed. The washing process was repeated twice and the precipitate after the washings was

dried and re-suspended with 10 ml of a solution 70:29.5:0.5 acetone/MilliQwater/formic acid (Sigma-Aldrich, Milan, Italy). The solution was vortex, centrifuged and clarified by filtration through 0.45 µm syringe filters (Labware, LLG, CA, USA) and then evaporated. The extract was re-suspended with 5 ml of MilliQ water and passed through a C18 cartridge (Sep-pack, USA). The column was washed with 5 ml of MilliQ water to recover the samples.

The HPLC system (Thermoquest Corporation, San Jose, CA, USA) was equipped with an isocratic pump (P1000), a multiple autosampler (AS3000) fitted with a 20 µL loop, a UV detector (UV100) set at 210 nm and a refractive index detector (Spectra System RI-150, Thermo Electro Corporation). The analyses of sugars (glucose, fructose and sucrose) were performed isocratically, at 0.6 ml min^{-1} and 80 °C, with a 300 × 7.8 mm i.d. cation exchange column (Aminex HPX-87P) equipped with a Cation Carbo-P Microguard cartridge (Bio-Rad Laboratories, Hercules, CA, USA). The analyses of organic acids (acetic, lactic, malic, succinic, oxalic, gluconic, tartaric, pyruvic, fumaric, and citric acid) were performed isocratically, at 0.8 ml min^{-1} and 60 °C, with a 300 × 7.8 mm i.d. cation exchange column (Aminex HPX-87H) equipped with a Cation H+ Microguard cartridge (Bio-Rad Laboratories, Hercules, CA, USA). The data treatments were carried out using the Chrom Quest™ chromatography data system (ThermoQuest, Inc., San Jose, CA, USA). Analytical grade reagents were used as standards (Sigma-Aldrich, St. Louis, MO). All samples were analyzed in triplicates of each biological replicate and the identification of compounds was performed by comparing the retention time of standard. Calibration curves of standards were performed by injecting serial dilutions of glucose, sucrose, fructose, acetic, lactic, malic, succinic, oxalic, gluconic, tartaric, pyruvic, fumaric and citric acid following the same conditions of the sample analyses. The concentration of the compounds was calculated by plotting a linear curve of the areas obtained in each sample.

Volatile metabolites produced by microbiota consortia. The dynamics of the volatile organic compounds (VOCs) of fermented cocoa beans-pulp under different conditions previously lyophilized were obtained using headspace solid phase micro-extraction

technique (HS- SPME), setting fiber conditions and oven temperatures as previously described by Rodriguez-Campos *et al.,* (2011) with some modifications (41). Samples were analyzed by triplicates of each biological replicate. The analysis was conducted in a 20 ml vial filled with 2 ml of 20 % NaCl and 0.1 g of the sample and adding to each sample 10 µl of 5-nonanol in ultrapure water at 50 mg/L concentration as an internal standard for the semi-quantification. The fiber with VOCs were injected into the Gas Chromatography-qMass Spectrometry (GC-qQP2010 Plus, Shimadzu, USA), equipped with an auto-sampler (AOC-5000, PAL System, CombiPAL, Switzerland) and a DB-WAXETR capillary column (30m × 0.25 mm, 0.25 µm film thickness, J&W Scientific Inc., Folsom, CA). The injection mode was established at 260 °C (1 min) using helium as carrier gas at a constant flow rate of 1 ml/min. The detection was carried out by the electron impact mass spectrometer in total ion current mode, using ionization energy of 70 eV. The acquisition range was settled as *m/z* 33-350 amu. The identification of the peaks was calculated by comparing the mass spectra of the peaks with the spectra of the MIST05 library and through comparison of the retention indices (a matrix of a homologous series of C8-C24 was used) with pure standard injected following the same sample conditions, described above. Semi-quantitative data (µg/kg) were obtained by measuring the characteristic *m*/*z* peak area of each identified compound in relation to the added internal standard.

Statistical analyses. Statistical analyses were carried out using generalized linear mixed-effects models for non-normally distributed data set. Mixed models were chosen for their ability to capture both fixed (fermentation condition: inoculated with S, ST and non-inoculated and fermentation time: 0-120 h) and random effects (fermentation type: B and H) (42). The *P*-values were adjusted using Bonferroni's method, when the linear mixed model revealed significant differences ($P < 0.05$) the Duncan honestly significant difference (HSD) test was applied. Mixed models were built and evaluated following Crawley (43) using R version 3.3.2

DNA extraction, library preparation, and sequencing. Total DNA was extracted from the pellet of cocoa matrices by using the MasterPure Complete DNA & RNA Purification kit (Illumina Inc, San Diego, CA) following the manufacturer's instructions. Bacterial communities were studied by amplifying the V3 and V4 region of the 16S rRNA using primers and condition described by Klintword *et al.* (44). Yeast communities were studied by amplifying the ITS2 region using ITS3tagmix (5'-CTAGACTCGTCACCGATGAAGAACGCAG) and ITS-4ngs (5'-TTCCTSCGGCTTATTGATATGC) (45). The PCR products were purified twice by means of an Agencourt AMPure kit (Beckman Coulter, Milan, Italy), and the resulting products were tagged by using the Nextera XT index kit (Illumina), according to the manufacturer's instructions. After the second clean up step with the Agencourt AMPure kit a 4nM pool was obtained taking into account the weight of the library measured by Qubit Fluorometric Quantitation (Thermo Fisher Scientific), and the mean amplicon size. A denaturated 20pmM pool was obtained mixing 5µl of NaOH 0,2N with 5 µl of the 4 nm pool. A final 10 pM library was combined with 10% PhiX. Sequencing was performed by MiSeq instrument (Illumina) with V3 chemistry and generated 250-bp paired-end reads, following the producer's instructions.

Bioinformatics. Paired-end reads were first assembled with FLASH software (46), with default parameters. Joint reads were further quality filtered (Phred < Q20) using the QIIME 1.9.0 software (47). Reads shorter than 250 bp were discarded by using Prinseq. For the 16S data, OTUs were picked at 99 % of similarity threshold and centroids sequences of each cluster were used to assign taxonomy by mapping against the Greengenes 16S rRNA gene database, version 2013 as described recently (48). Chloroplast and mitochondria sequences were removed from the dataset. For the ITS dataset, 97 % of similarity was picked for OTUs by means of UCLUST clustering methods (49), and representative sequences of each cluster were used to assign taxonomy using the UNITE rDNA ITS database version 2012, by means of the RDP Classifier. Weighted and Unweighted UniFrac distance matrices,

as well as OTUs table, were used to find differences between fermentation processes (B and H) and under different conditions (inoculated and non-inoculated) by Adonis and Anosim statistical test in R environment In order to avoid biases due to different sequencing depths, all samples for each dataset were rarefied at the lowest number of reads after raw read quality filtering. QIIME was used to produce a filtered OTU table at 1 % in at least 2 samples. The OTU table displays the higher taxonomy resolution reached when the taxonomy assignment was not able to reach the species level, genus or family name was displayed. The statistical package Kruskal–Wallis and Mann–Whitney tests were used to find significant differences ($P < 0.05$) in microbial taxa abundance profiles and the Shannon-Wiener diversity index *H'* according to time, conditions and methods. As a measure of the association between microbial OTUs occurring in at least 2 samples and chemical variables, the Spearman's rank correlation coefficient was obtained through the function *psych* and plotted through the *corrplot* package of R. OTUs occurring in at least 2 samples from microbial communities were conglomerate by hierarchical clustering analysis using Ward's method acquired thorough the function *heatplot* plotted through the *made4* package of R.

Accession number(s). The 16S and ITS rRNA gene sequences are available at the Sequence Read Archive of the NCBI (accession number SRP126069 and SRP12608 respectively

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709 **TABLE LEGEND**

710

711 **Table 1.** Average changes in physical and microbiological parameters during inoculated and
712 non-inoculated Box and Heap fermentation of cocoa bean-pulp turned after 48 and 96 h

713

714 **Table 2.** Incidence of the fungal taxonomic groups by amplicon sequencing expressed as
715 relative abundances. Only OTUs with an incidence above 1 % in at least 2 samples are shown

716

717 **Table 3.** Occurrence of the bacterial taxonomic groups by amplicon sequencing expressed as
718 relative abundances. Only OTUs with an incidence above 1 % in at least 2 samples are shown

719

720

FIGURE LEGEND

Fig 1. Dynamics of sugars and organic acid compounds in cocoa bean-pulp inoculated and non-inoculated during Box and Heap fermentations expressed as mg/g. Data are expressed as mean ± SD values from triplicate determinations

Fig 2. Spearman's correlation between microbial OTUs observed with an incidence above >1 % in at least 2 samples. Samples are label according to fermentation method **A)** Box and **B)** Heap. Rows and columns are clustered by Ward linkage hierarchical clustering. The intensity of the colors represents the degree of correlation between fungal and bacterial OTUs as measured by the Spearman's correlation. The intensity of the colors represents the degree of correlation between yeast and bacterial with blue (negative degree of correlation) and red (positive degree of correlation).

Fig 3. Correlation plot showing Spearman's correlation between microbial OTUs and metabolites observed with an incidence above > 1% in at least 2 samples. Samples are label according to fermentation method **A)** Box and **B)** Heap. Significance associations between OTUs and metabolites are only shown ($P < 0.05$). The intensity of the colors represents the degree of correlation between fungal and bacterial OTUs as measured by the Spearman's correlation, were the color blue represents a positive degree of correlation and red a negative correlation between sugars, organic acids and OTUs

745 **Table 1.**

		FERMENTATION TIME (h)			
		0	**48**	**96**	**120**
BOX					
°C	S	26.73 ± 0.60	36.20 ± 1.53	41.88 ± 1.78	43.70 ± 2.94
	ST	26.48 ± 0.34	35.10 ± 2.46	41.73 ± 2.06	42.78 ± 3.68
	C	26.73 ± 0.62	36.20 ± 1.53	41.88 ± 1.78	43.70 ± 2.94
	A	***26.58 ± 0.08***^{c}	***35.80 ± 1.22***^{b}	***42.09 ± 0.50***^{a}	***43.33 ± 0.70***^{a}
pH	S	3.55 ± 0.03	3.88 ± 0.16	4.15 ± 0.11	3.96 ± 0.18
	ST	3.54 ± 0.01	4.00 ± 0.16	4.20 ± 0.15	4.18 ± 0.26
	C	3.55 ± 0.03	3.88 ± 0.17	4.27 ± 0.11	3.96 ± 0.18
	A	***3.57 ± 0.03***^{c}	***4.08 ± 0.11***^{b}	***4.31 ± 0.09***^{a}	***4.15 ± 0.17***^{ab}
Yeast (Log CFU)	S	7.08 ± 0.05	7.19 ± 0.15	N.C	N.C
	ST	7.14 ± 0.01	7.05 ± 0.06	N.C	N.C
	C	7.19 ± 0.17	7.55 ± 0.27	N.C	N.C
	A	***7.14 ± 0.11***^{a}	***7.26 ± 0.29***^{a}	*N.C*	*N.C*
LAB (Log CFU)	S	5.38 ± 0.30	7.18 ± 0.02	5.11 ± 1.28	6.75 ± 0.17
	ST	6.13 ± 0.22	6.85 ± 0.27	4.33 ± 0.38	7.35 ± 0.25
	C	6.21 ± 0.74	6.88 ± 0.40	4.00 ± 0.00	5.49 ± 0.59
	A	***5.91 ± 0.61***^{b}	***6.97 ± 0.31***^{a}	***5.44 ± 2.85***^{c}	***6.55 ± 0.94***^{a}
AAB (Log CFU)	S	6.41 ± 0.11	7.01 ± 0.12	5.54 ± 1.78	5.63 ± 1.88
	ST	6.28 ± 0.21	6.96 ± 0.06	5.60 ± 1.84	7.34 ± 0.09
	C	6.28 ± 0.25	7.31 ± 0.26	4.00 ± 0.00	5.76 ± 0.49
	A	***6.32 ± 0.20***^{a}	***7.09 ± 0.23***^{a}	***5.05 ± 2.01***^{b}	***6.69 ± 2.85***^{a}
HEAP					
°C	S	28.20 ± 1.15	38.17 ± 0.75	36.57 ± 1.80	40.07 ± 0.12
	ST	27.37 ± 0.32	39.00 ± 2.21	36.57 ± 0.90	39.38 ± 0.32
	C	26.27 ± 0.06	38.97 ± 0.32	39.37 ± 2.57	40.30 ± 0.56
	A	***27.28 ± 0.97***^{c}	***38.71 ± 0.47***^{b}	***38.40 ± 1.59***^{b}	***40.07 ± 0.23***^{a}
pH	S	3.55 ± 0.01	4.24 ± 0.17	4.48 ± 0.79	4.90 ± 0.97
	ST	3.53 ± 0.01	4.32 ± 0.23	4.05 ± 0.40	4.52 ± 0.74

	C	3.50 ± 0.05	3.87 ± 0.08	4.24 ± 0.29	3.99 ± 0.33
	A	***3.54 ± 0.02***^{b}	***4.28 ± 0.24***^{a}	***4.26 ± 0.21***^{a}	***4.71 ± 0.90***^{a}
Yeast (Log CFU)	S	7.16 ± 0.92	7.80 ± 0.15	7.13 ± 0.16	8.03 ± 0.29
	ST	6.76 ± 0.85	7.72 ± 0.15	7.24 ± 1.41	7.43 ± 0.07
	C	7.02 ± 0.71	6.62 ± 0.02	6.34 ± 0.04	7.24 ± 0.28
	A	***6.98 ± 0.20***^{c}	***7.38 ± 0.66***^{b}	***6.90 ± 0.48***^{d}	***7.57 ± 0.41***^{a}
LAB (Log CFU)	S	5.67 ± 0.25	7.28 ± 0.19	7.36 ± 0.04	7.69 ± 0.28
	ST	5.95 ± 0.29	7.07 ± 0.09	7.00 ± 0.01	7.50 ± 0.04
	C	5.72 ± 0.03	6.17 ± 0.21	7.40 ± 0.04	8.10 ± 0.13
	A	***5.78 ± 0.15***^{d}	***6.84 ± 0.59***^{c}	***7.25 ± 0.22***^{b}	***7.76 ± 0.30***^{a}
AAB (Log CFU)	S	6.20 ± 0.23	6.81 ± 0.14	8.33 ± 0.02	7.80 ± 0.18
	ST	5.70 ± 0.04	7.15 ± 0.28	8.08 ± 0.11	7.66 ± 0.01
	C	6.60 ± 0.04	5.65 ± 0.15	8.42 ± 0.02	8.56 ± 0.04
	A	***6.17 ± 0.45***^{d}	***6.54 ± 0.79***^{c}	***8.28 ± 0.18***^{a}	***8.00 ± 0.49***^{b}

Values are expressed as the mean ± SD from triplicate determinations. **Abbreviations**: **S:** *S. cerevisiae*, **ST:** *S. cerevisiae* and *T. delbrueckii*, **C:** Non-inoculated, **A:** Average N.C: Below detection limit. Different letters indicate statistical difference related to the fermentation time period using least significant difference test ($P < 0.05$). *P*-values were adjusted using Bonferroni's method.

749 **Table 2.**

	Box				Heap				Box				Heap				Box				Heap			
	T0	**T48**	**T96**	**T120**	**T0**	**T48**	**T96**	**T120**	**T0**	**T48**	**T96**	**T120**	**T0**	**T48**	**T120**		**T0**	**T48**	**T96**	**T120**	**T0**	**T48**	**T96**	**T120**
	S. cerevisiae								***S. cerevisiae + T. delbruecki***								**Non-inoculated**							
Botryosphaeria	1.33	0.43	0.46	0.21	0.59	1.55	0.13	0.13	0.53	0.44	0.91	3.51	0.56	0.32	0.59		0.66	0.20	1.08	0.16	11.07	0.00	0.47	0.12
Candida	3.33	1.35	1.21	1.27	1.03	0.46	0.31	0.74	1.89	0.97	0.77	5.56	0.53	0.34	0.72		2.83	1.48	2.59	0.49	0.89	0.24	1.39	1.73
Candida butyri	2.23	0.27	0.42	0.50	0.16	0.03	0.07	0.09	0.77	0.69	0.34	0.40	0.15	0.04	0.07		0.70	0.21	1.16	0.09	0.07	0.00	0.07	0.03
Candida diversa	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	5.50	0.00	0.00	0.00		0.00	1.00	0.96	0.00	0.00	0.00	0.00	0.00
Candida inconspicua	0.00	0.58	2.63	7.82	0.06	0.03	0.25	0.32	0.00	0.74	1.51	12.97	0.00	0.04	0.94		0.03	2.61	0.96	13.47	0.00	0.87	1.62	1.43
Candida jaroonii	13.07	4.40	3.63	1.24	4.01	1.65	0.59	1.00	8.06	3.80	2.29	1.24	3.41	1.55	1.96		4.34	2.18	3.04	0.86	1.89	0.24	1.27	0.80
Candida quercitrusa	2.27	1.03	0.78	0.32	1.31	0.94	0.32	0.81	1.40	0.72	1.17	0.49	1.27	0.99	1.34		2.43	1.08	1.04	0.16	0.47	0.09	1.46	0.89
Ceratocystis	3.08	5.98	2.93	1.42	1.43	0.99	0.50	0.50	2.46	2.91	2.91	3.41	1.27	1.56	2.32		4.19	2.45	9.32	4.81	2.07	0.87	1.53	0.80
Hanseniaspora opuntiae	38.33	40.98	39.72	28.21	49.76	39.10	48.67	64.37	36.78	37.25	15.62	0.38	43.73	29.51	11.70		54.21	48.11	29.13	21.78	69.22	72.35	35.05	44.81
Issatchenkia	0.09	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.27	0.25	0.00	0.37		0.00	0.41	0.37	0.00	0.00	0.00	2.46	1.18
Kluyveromyces marxianus	0.00	0.28	0.18	8.91	0.06	0.13	11.18	1.79	0.00	0.42	0.38	15.76	0.00	0.13	2.80		0.01	4.61	1.03	43.54	0.09	7.76	7.23	1.98
Lasiodiplodia theobromae	0.83	0.61	2.07	1.33	0.49	0.49	0.09	0.07	3.30	0.91	1.22	0.90	0.80	0.09	0.25		4.94	0.61	0.92	0.27	0.10	0.16	0.07	0.16
Penicillium	0.62	0.39	1.09	0.16	0.19	0.13	0.00	0.06	0.12	0.16	0.31	0.25	0.10	0.19	0.27		0.55	0.12	0.24	0.10	0.15	0.02	0.52	0.21
Pichia	0.56	0.09	0.05	0.16	0.12	0.12	0.01	0.78	0.06	0.09	0.15	0.60	0.07	0.01	0.49		0.37	0.41	0.55	0.19	0.19	0.00	1.21	1.28
Pichia pijperi	10.90	10.23	12.75	15.36	10.21	14.55	6.79	8.42	7.67	9.57	9.05	18.01	13.62	18.35	17.13		11.32	10.91	14.24	8.38	10.37	6.29	29.42	24.14
Saccharomyces cerevisiae	12.95	28.62	28.30	30.22	26.08	37.67	30.29	19.11	7.73	21.50	31.97	15.98	7.67	21.57	33.96		0.28	19.95	27.12	3.35	0.37	10.28	11.92	17.96
Saccharomycopsis	0.72	0.35	0.32	0.15	0.13	0.15	0.07	0.13	1.12	0.30	0.32	0.03	0.27	0.10	0.32		0.82	0.49	1.21	0.22	0.52	0.13	0.32	0.34
Torulaspora delbrueckii	0.06	0.01	0.04	0.01	0.03	0.00	0.01	0.04	25.35	16.32	28.18	8.48	23.02	20.45	22.50		0.02	0.07	0.27	0.03	0.12	0.09	0.13	0.09

750 Values are expressed as the mean from duplicate determinations. The abundance of OTUs in the 2 biological replicates of each sampling time was averaged. Samples are label according to fermentation time (0, 48, 96
751 and 120 h), fermentation method (Box and Heap), and condition (inoculated with S, ST and non-inoculated).

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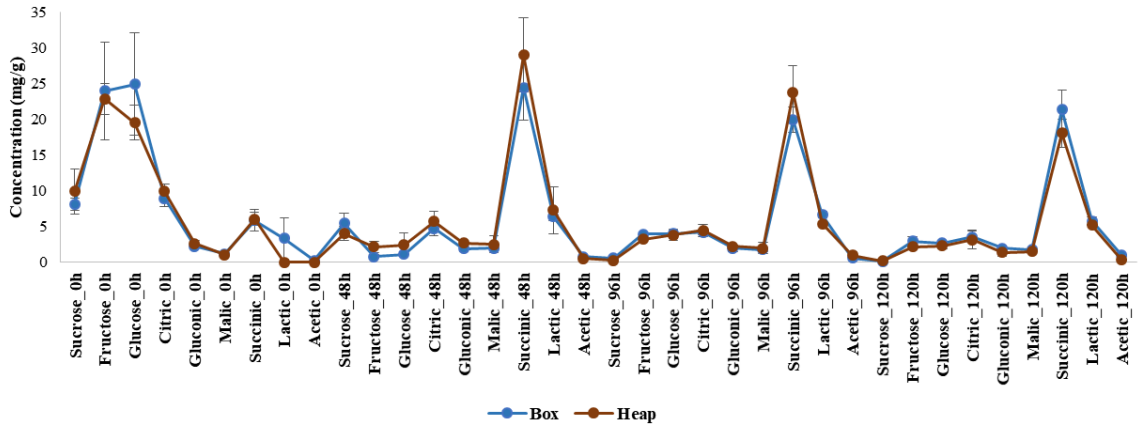
Table 3.

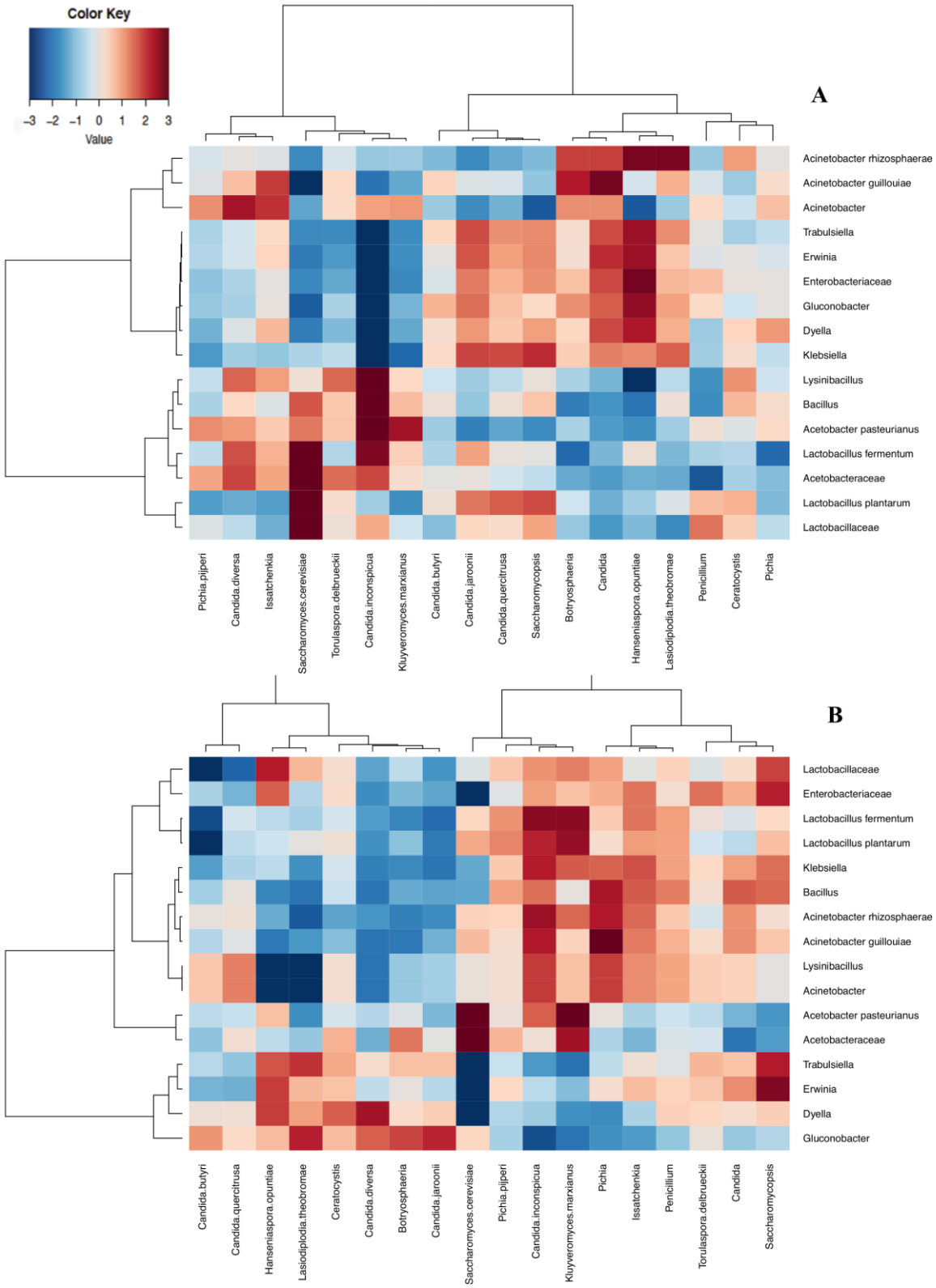
	Box				Heap				Box				Heap				Box				Heap			
	T0	**T48**	**T96**	**T120**	**T0**	**T48**	**T96**	**T120**	**T0**	**T48**	**T96**	**T120**	**T0**	**T48**	**T96**	**T120**	**T0**	**T48**	**T96**	**T120**	**T0**	**T48**	**T96**	**T120**
	S. cerevisiae								***S. cerevisiae + T. delbrueckii***								**Non-inocualted**							
Acetobacter pasteurianus	3.16	13.81	62.90	52.83	1.05	40.27	76.99	69.22	3.02	42.67	63.42	86.96	0.96	57.24	79.19	24.74	2.40	18.22	42.67	64.24	4.70	28.52	36.67	45.93
Acetobacteraceae	0.19	1.73	1.01	1.29	0.00	16.87	6.23	5.08	0.29	4.70	0.86	1.05	0.10	11.79	4.41	0.86	0.10	0.58	3.45	2.40	0.38	5.42	1.44	0.10
Acinetobacter	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.29	0.00	0.00	0.05	0.00	0.00	0.00	0.00	4.99	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00
Acinetobacter guillouiae	0.24	0.00	0.00	0.00	0.00	0.00	0.00	0.86	0.10	0.00	0.10	0.00	0.00	0.00	0.00	1.25	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.14
Acinetobacter rhizosphaerae	0.00	0.00	0.00	0.00	0.00	0.00	0.19	1.44	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.25	0.00	0.00	0.00	0.00	0.05	0.00	0.19	0.24
Bacillus	0.00	0.00	0.00	16.35	0.00	0.00	0.00	0.77	0.00	0.00	11.94	0.29	0.00	0.00	0.00	3.36	0.00	0.10	0.00	4.41	0.05	0.00	0.96	8.96
Dyella	1.68	0.58	0.10	0.14	0.77	0.10	0.00	0.00	0.43	0.38	0.10	0.00	0.19	0.19	0.00	0.00	1.25	0.29	0.58	0.19	0.96	1.10	0.53	0.14
Enterobacteriaceaee	3.26	1.34	1.20	0.29	0.00	0.00	0.00	0.10	0.96	0.58	0.10	0.00	0.19	0.00	0.00	0.10	1.82	1.53	0.58	0.19	2.21	1.53	0.62	0.34
Erwinia	4.94	1.68	1.10	0.53	0.19	0.19	0.00	0.00	1.63	0.72	0.19	0.00	0.29	0.10	0.00	0.00	3.45	1.53	0.77	0.58	2.30	1.82	1.05	0.34
Gluconobacter	3.36	1.25	0.38	0.05	1.63	1.34	0.38	0.19	4.07	1.15	0.05	0.10	2.97	1.25	0.38	0.10	2.40	0.67	0.58	0.00	2.88	1.34	0.10	0.10
Klebsiella	0.34	0.43	0.19	0.24	0.00	0.00	0.19	0.19	1.10	0.19	0.10	0.00	0.00	0.00	0.10	0.48	1.25	1.15	0.48	0.10	0.38	0.34	0.34	3.93
Lactobacillaceae	0.05	2.06	3.88	1.20	0.00	0.38	0.00	0.10	0.05	1.68	0.96	0.00	0.00	0.10	0.19	0.10	0.10	5.37	2.21	1.25	0.19	0.62	1.10	0.34
Lactobacillus plantarum group	1.44	15.58	12.27	4.31	0.00	2.11	0.38	0.19	0.91	10.74	1.92	0.67	0.00	1.15	0.19	1.63	0.58	28.57	14.09	5.47	0.29	3.74	6.62	1.49
Lactobacillus fermentum	0.19	31.59	3.40	8.63	0.00	10.07	7.19	5.75	0.14	6.14	10.12	1.44	0.00	9.20	6.90	12.18	0.10	3.55	0.58	7.00	0.05	30.87	13.71	16.73
Lysinibacillus	0.00	0.00	0.00	0.58	0.00	0.00	0.00	0.67	0.00	0.00	1.63	0.00	0.00	0.00	0.00	0.96	0.00	0.00	0.00	0.67	0.00	0.00	0.19	0.05
Trabulsiella	5.13	3.12	2.49	1.53	0.38	0.29	0.00	0.00	2.64	0.81	0.53	0.19	0.58	0.10	0.00	0.10	5.75	1.73	1.53	0.86	2.97	4.12	0.96	0.38

Values are expressed as the mean from duplicate determinations. The abundance of OTUs in the 2 biological replicates of each sampling time was averaged. Samples are label according to fermentation time (0, 48, 96 and 120 h), fermentation method (Box and Heap), and condition (inoculated with S, ST and non-inoculated)

Fig. 1

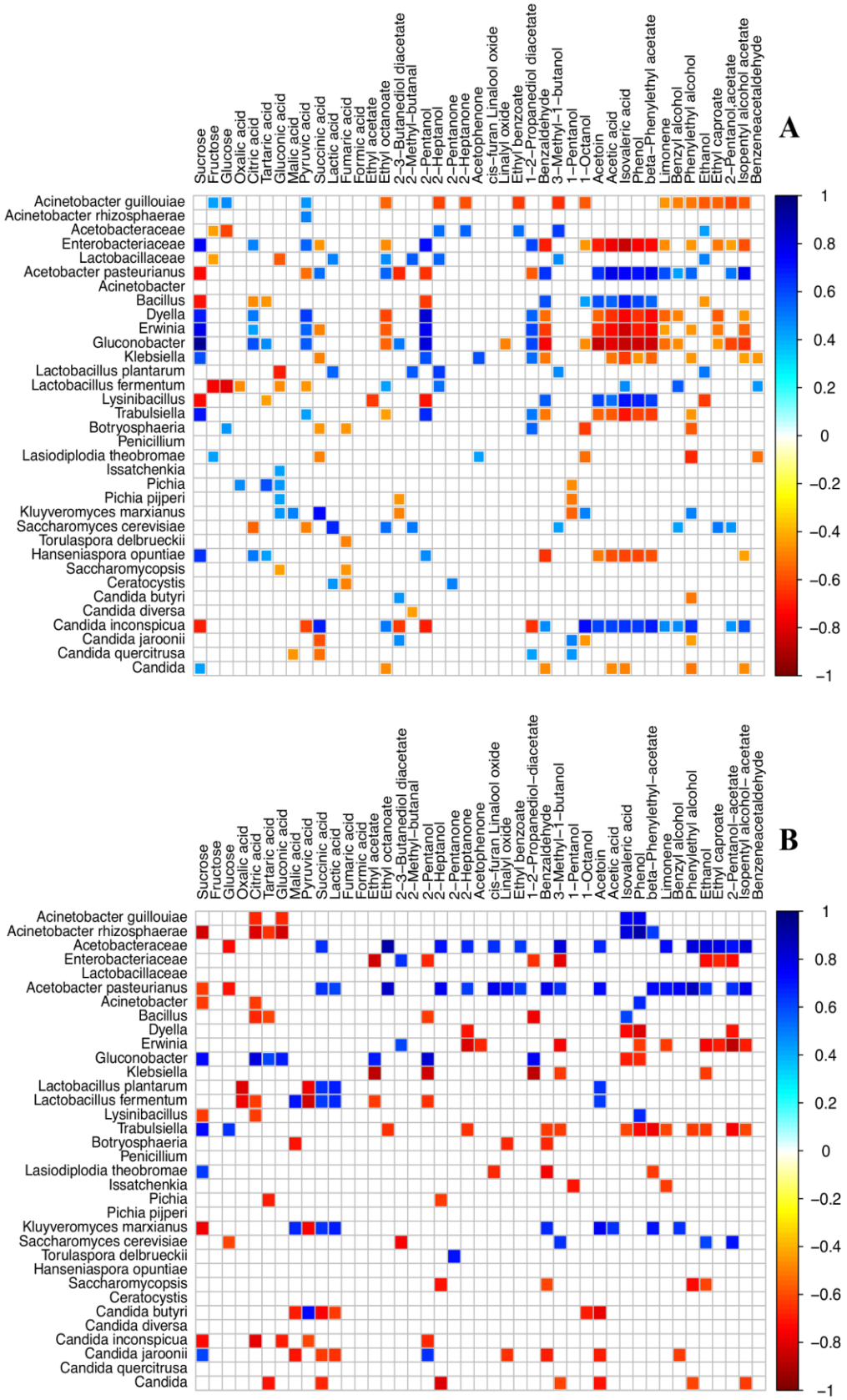

Concentration (mg/g)
35
30
25
20
15
10
5
0
Sucrose_0h
Fructose_0h
Glucose_0h
Citric_0h
Gluconic_0h
Malic_0h
Succinic_0h
Lactic_0h
Acetic_0h
Sucrose_48h
Fructose_48h
Glucose_48h
Citric_48h
Gluconic_48h
Malic_48h
Succinic_48h
Lactic_48h
Acetic_48h
Sucrose_96h
Fructose_96h
Glucose_96h
Citric_96h
Gluconic_96h
Malic_96h
Succinic_96h
Lactic_96h
Acetic_96h
Sucrose_120h
Fructose_120h
Glucose_120h
Citric_120h
Gluconic_120h
Malic_120h
Succinic_120h
Lactic_120h
Acetic_120h
Box
Heap

760 **Fig. 2**



761

762


A
Sucrose
Fructose
Glucose
Oxalic acid
Citric acid
Tartaric acid
Gluconic acid
Malic acid
Pyruvic acid
Succinic acid
Lactic acid
Fumaric acid
Formic acid
Ethyl acetate
Ethyl octanoate
2–3–Butanediol diacetate
2–Methyl–butanal
2–Pentanol
2–Heptanol
2–Pentanone
2–Heptanone
Acetophenone
cis–furan Linalool oxide
Linalyl oxide
Ethyl benzoate
1–2–Propanediol diacetate
Benzaldehyde
3–Methyl–1–butanol
1–Pentanol
1–Octanol
Acetoin
Acetic acid
Isovaleric acid
Phenol
beta–Phenylethyl acetate
Limonene
Benzyl alcohol
Phenylethyl alcohol
Ethanol
Ethyl caproate
2–Pentanol,acetate
Isopentyl alcohol acetate
Benzeneacetaldehyde
Acinetobacter guillouiae
Acinetobacter rhizosphaerae
Acetobacteraceae
Enterobacteriaceae
Lactobacillaceae
Acetobacter pasteurianus
Acinetobacter
Bacillus
Dyella
Erwinia
Gluconobacter
Klebsiella
Lactobacillus plantarum
Lactobacillus fermentum
Lysinibacillus
Trabulsiella
Botryosphaeria
Penicillium
Lasiodiplodia theobromae
Issatchenkia
Pichia
Pichia pijperi
Kluyveromyces marxianus
Saccharomyces cerevisiae
Torulaspora delbrueckii
Hanseniaspora opuntiae
Saccharomycopsis
Ceratocystis
Candida butyri
Candida diversa
Candida inconspicua
Candida jaroonii
Candida quercitrusa
Candida
1
0.8
0.6
0.4
0.2
0
−0.2
−0.4
−0.6
−0.8
−1
B
Sucrose
Fructose
Glucose
Oxalic acid
Citric acid
Tartaric acid
Gluconic acid
Malic acid
Pyruvic acid
Succinic acid
Lactic acid
Fumaric acid
Formic acid
Ethyl acetate
Ethyl octanoate
2–3–Butanediol diacetate
2–Methyl–butanal
2–Pentanol
2–Heptanol
2–Pentanone
2–Heptanone
Acetophenone
cis–furan Linalool oxide
Linalyl oxide
Ethyl benzoate
1–2–Propanediol–diacetate
Benzaldehyde
3–Methyl–1–butanol
1–Pentanol
1–Octanol
Acetoin
Acetic acid
Isovaleric acid
Phenol
beta–Phenylethyl–acetate
Limonene
Benzyl alcohol
Phenylethyl alcohol
Ethanol
Ethyl caproate
2–Pentanol–acetate
Isopentyl alcohol– acetate
Benzeneacetaldehyde
Acinetobacter guillouiae
Acinetobacter rhizosphaerae
Acetobacteraceae
Enterobacteriaceae
Lactobacillaceae
Acetobacter pasteurianus
Acinetobacter
Bacillus
Dyella
Erwinia
Gluconobacter
Klebsiella
Lactobacillus plantarum
Lactobacillus fermentum
Lysinibacillus
Trabulsiella
Botryosphaeria
Penicillium
Lasiodiplodia theobromae
Issatchenkia
Pichia
Pichia pijperi
Kluyveromyces marxianus
Saccharomyces cerevisiae
Torulaspora delbrueckii
Hanseniaspora opuntiae
Saccharomycopsis
Ceratocystis
Candida butyri
Candida diversa
Candida inconspicua
Candida jaroonii
Candida quercitrusa
Candida
1
0.8
0.6
0.4
0.2
0
−0.2
−0.4
−0.6
−0.8
−1

763 Fig. 3