Dynamics and biodiversity of bacterial and yeast communities during the fermentation of cocoa beans

Jatziri Mota-Gutierrez[1], Cristian Botta[1], Ilario Ferrocino[1], Manuela Giordano[1], Marta Bertolino[1], Paola Dolci[1], Marcella Cannoni[2], Luca Cocolin*[1]

[1]Department of Agricultural, Forest, and Food Science, University of Turin, Largo Paolo Braccini 2, 10095, Grugliasco, Torino, Italy

[2] SOREMARTEC ITALIA S.r.l., Piazza Ferrero 1 – 12051, Alba, Cuneo, Italy

*Corresponding author: Luca Cocolin, lucasimone.cocolin@unito.it

Running title: Development of bacteria and yeast in cocoa fermentation

ABSTRACT

Forastero hybrid cocoa bean fermentations have been carried out in a Box (B) and in a Heap (H), with or without the inoculation of *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* as starter cultures. The bacteria, yeasts and microbial metabolites (volatile and non-volatile organic compounds) were monitored during fermentation to assess the connection between microbiota and the release of metabolites during this process. The presence of starter cultures was detected, by means of culture-dependent analysis, during the first two days of both fermentations. However, no statistical difference was observed in any of the physico-chemical or microbiological analyses. Plate counts revealed the dominance of yeasts at the beginning of both fermentations, and these were 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), although different relative abundances were observed. Only the diversity of the fungal species indicated a higher level of complexity in the B fermentations than in the H fermentations ($P < 0.05$) as well as a statistically significant difference between the initially inoculated starter cultures ($P < 0.01$). However, the microbial metabolite analysis indicated different distributions of the volatile and non-volatile compounds between the two procedures, that is, B and H ($P < 0.05$), rather than between the inoculated and non-inoculated fermentations. The Box fermentations showed a faster carbohydrate metabolism and greater production of organic acid compounds, which boosted the formation of alcohols and esters, than the heap fermentations. Overall, the microbial dynamics and associations between the bacteria, yeast and metabolites were found to depend on the type of fermentation.

IMPORTANCE

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

available suggests that microbial communities have played an important role in the evolution of aroma compounds. Understanding the pathways that micro-organisms follow during the formation of aromas could be used to improve the fermentation processes and to enhance chocolate quality.

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

INTRODUCTION

Cocoa (*Theobroma cacao* L.) is an important plant crop throughout the world and its production serves as a main source of income in several developing countries (1). According to the Food and Agriculture Organization (FAO), the world cocoa bean production was 4,466,574 tonnes in 2016 (2). In terms of overall amount of beans per country, the main cocoa-producing countries in 2016 were the Ivory Coast, followed by Ghana, Indonesia and Cameroon (2). Chocolate production begins with the harvesting of 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). Spontaneous fermentation normally lasts from 3 to 10 days in heaps, boxes, baskets or trays.

According to Schwan and Fleet (4), the microbiota present during cocoa fermentation is composed of yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB). Two important stages are present during cocoa fermentation; in the first stage, yeasts proliferate in the reducing sugars and citric acid from the pulp and produce ethanol and carbon dioxide. At the same time, the temperatures and pH increase, due to aerobic and oxidative reactions, thus allowing LAB and AAB to grow (4). LAB mainly transforms sugars and organic acids 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 an increase in temperatures (3). The quality of the end product chocolate depends on the three previously cited groups of microorganisms, since they are able to produce metabolites and flavor precursors (4).

Although the importance of yeasts during cocoa fermentation has emerged in recent studies (5, 6), fungal biodiversity in fermented food has been studied far less than bacteria. In spite of the use of high-throughput sequencing (HTS) this decade, this new technology has mainly been used to obtain new insights into the domain of fermented foods as it enables the genetic variants of a complex ecosystem to be discovered, validated and screened (7). The

importance of identifying the microbial composition of food ecosystems involves finding appropriate starter cultures that enhance a particular aspect of the product. *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* have recently been detected and used as starter cultures in cocoa fermentation, and they have shown a positive impact on the aroma profile of the end product (8, 9). However, there has been much controversy concerning the choice of the starter cultures used in cocoa fermentation to improve the quality of the end product. This paper explores the impact of two cocoa fermentation starter cultures introduced to guarantee the production of cocoa beans with specific and reproducible features exposed to different fermentation methods. We point out the challenges relate to the reproducibility of the effect of the starter cultures on cocoa bean fermentations and their correlations with the initial microbial populations and importance on aroma development.

The present study has been aimed at determining the dynamics and biodiversity of both bacteria and yeasts by means of amplicon-based sequencing of the 16S rRNA genes and the ITS2 gene, respectively, during cocoa bean fermentation carried out both spontaneously and in the presence of yeast starter cultures, in both boxes and heaps in order to acquire more detailed knowledge about the relationship between microorganisms and their surroundings. The non-volatile and volatile organic compounds were also assessed with the aim of investigating how the use of cultures can affect the volatilome profile of fermented cocoa from the two different fermentation processes. In this study, we have also proposed the measurements of associations between microbial communities and the development of microbial volatile and non-volatile compounds. A better understanding of the microbial communities and physico-chemical dynamics during box and heap fermentations will undoubtedly help to develop new management procedures for the production of high-quality cocoa.

RESULTS

The physical and microbiological changes that take place in box and heap fermentations. The temperature and pH were measured during the 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 considered conditions (inoculated and non-inoculated) was observed from the physical or microbiological analysis, while the temperature observed during the B and H fermentations increased significantly from the initial values of 27 °C to 43 °C and 40 °C, respectively, at the end of the fermentation ($P < 0.05$). The pH of the cocoa bean-pulp was 3.5 at the beginning of the trial, and it increased to 4.2 and 4.7 at the end of the fermentation for the B and H fermentations, respectively ($P < 0.05$).

The yeast, LAB and AAB population dynamics are reported in Table 1. The yeasts constituted the dominant population for the first 48 hours in both processes (B and H), and they were already detected at high loads in the cocoa beans before the introduction of the starter strain inoculum, with an average value of 6.98 log CFU g^{-1} in the H and 7.14 log CFU g^{-1} in the B fermentations. On the other hand, the yeast population in the H fermentation remained at around 7 log CFU g^{-1}, even after 48 hours, with the highest count recorded at the end of the process (7.57 log CFU g^{-1}). A significant difference between B and H was also observed in the LAB dynamics during the fermentation time, with a marked increase in 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 the B and H fermentations (6.32 and 6.17 log CFU g^{-1}, respectively). However, this population showed a fluctuating trend during the B fermentation, whereas, it increased over time during the H fermentation to final counts of 8.00 log CFU g^{-1} ($P < 0.01$). It should be noted that, after 96 hours, the AAB dominated the LAB and yeasts in both fermentation processes. Overall, higher counts were observed for the three considered microbial groups (yeasts, LAB and AAB) in the H fermentations than in the B ones at 96 hours, as shown in Table 1 ($P < 0.05$).

Identification of isolated yeast colonies and assessment of the dominance of the starter strains. In order to establish the yeast dynamics in the 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 a comparison with the starter profiles highlighted the presence of *S. cerevisiae* ID76 and *T. delbrueckii* ID103 in the cultivable mycobiota during the first 48 hours of both the B and H fermentations. *S. cerevisiae* ID76 represented 68 % of the isolates from the fermentations inoculated with *S. cerevisiae* (S) and 51 % of the colonies isolated from the fermentations inoculated with *S. cerevisiae* and *T. delbrueckii* (ST). Finally, 38 % of the colonies isolated from the ST fermentations were ascribed to a *T. delbrueckii* ID103 profile. Apart from the identification of the starter strains, *Hanseniaspora opuntiae* represented the most abundant autochthonous species, representing 31 % of the colonies isolated from the non-inoculated fermentations (data not shown).

Dynamics of the non-volatile organic compounds during cocoa bean fermentation. The evolution of non-volatile compounds was determined during the B and H fermentations of the cocoa beans by means of High-Performance Liquid Chromatography (HPLC), as shown in Figure 1. No significant differences were observed between the inoculated and non-inoculated fermentations through the analysis of the non-volatile compounds. At the beginning of the process, the B fermentations showed higher concentrations of glucose, fructose and sucrose (24, 25 and 8 mg/g, respectively) than the H fermentations (20, 23 and 10 mg/g, respectively) and significantly decreased levels of glucose, fructose and sucrose during both fermentation processes (B and H) were observed over the fermentation period ($P < 0.05$).

As far as the overall content of organic acids is concerned, the highest concentration in the cocoa bean-pulp before the start of the fermentation was that of citric acid, and this was followed by succinic and gluconic acid in both fermentation processes (Fig. 1). It should be pointed out that higher amounts of lactic and succinic acid were detected at 48 hours, whereas the maximum production of acetic acid was observed at 96 hours. Succinic acid was found to be the most abundant organic acid, from 48 hours to the end of both fermentation processes (B and H), with concentrations of up to 21 and 18 mg/g, respectively. The dynamics over time observed for the organic acids during both fermentation processes (B and H) were similar. A statistically significant decrease in the citric and gluconic acid concentrations was observed

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

Volatilome during cocoa bean-pulp fermentation. A total of 72 volatile organic compounds (VOCs) were identified by means of 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 were observed between the inoculated and non-inoculated fermentations from the VOC analysis. At the beginning of the B and H fermentation processes, 2-pentanol, ethyl acetate, limonene and 1,2-propanediol diacetate were found to be the most abundant volatile compounds, whereas acetic acid, limonene, 2-heptanol, phenylethyl alcohol, isopentyl alcohol, isovaleric acid and benzeneacetaldehyde represented the most retrieved VOCs in the headspace at the end of both fermentations (see Table S1). It should be pointed out that the total peak area of the VOCs at the end of the B fermentation was about twice as high as that of the H fermentation (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 of the samples, with an ESC average of 97 % (see Table S2), while the alpha-diversity indicated a higher level of complexity in the B fermentations than in the 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 the non-inoculated fermentation (46.23 %) compared to those inoculated with with

195 *S. cerevisiae* and *T. delbrueckii* (ST) (25.60 %, $P < 0.05$). In addition, a significantly higher
196 presence of *T. delbrueckii* was observed in the fermentations inoculated with the mixed yeast
197 culture (ST, 22.23 %) than in the fermentations inoculated only with *S. cerevisiae* (S) and than
198 the non-inoculated ones (0.03 and 0.11 %, respectively) ($P < 0.01$, Table 2).

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

Overall, the most abundant OTUs detected at 48 hours in both the inoculated and non-inoculated B fermentations were *Acetobacter pasteurianus, Lactobacillus fermentum* and *L. plantarum* (Fig. 3). It should be noted that *A. pasteurianus* and *L. fermentum* remained the two most abundant OTUs at the end of the box fermentation for both conditions (inoculated or non-inoculated), and these were followed by *Bacillus*. As far as the inoculated H fermentations are concerned, *A. pasteurianus, L. fermentum* and *Acetobacteraceae* were the most abundant OTUs detected at 48 hours, and *A. pasteurianus* and *L. fermentum* remained the dominant OTUs over the entire fermentation period (Table 3). Instead, the non-inoculated H fermentations were characterized by a high relative abundance of *L. fermentum, A. pasteurianus*, and *L. plantarum* at 48 hours, while *L. fermentum, Bacillus* and *Klebsiella* took over and dominated at the end of the process. As far as the dynamics are concerned, we observed an increase in the relative abundances under different conditions for *L. fermentum, L. plantarum, A. pasteurianus, Bacillus, Acetobacteraceae* and *Lactobacillaceae* over the fermentation period, while *Erwinia, Gluconobacter, Trabulsiella* and *Enterobacteriaceaee* decreased over time ($P < 0.01$), as shown in Table 3.

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

Overall, *L. plantarum, A. pasteurianus* and *Enterobacteriaceaee* were negatively associated with the main yeast OTUs (*S. cerevisiae, K. marxianus, C. inconspicua* and *P. pijperi)* in the B fermentations. In short*, 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* ($P < 0.05$). It is worth noting that

H. opuntiae and *C. jaronii* were found to be positively associated with the minor OTUs, *Citrobacter* and *Erwinia* ($P < 0.05$, Figure 2A).

The *L. fermentum* in the H fermentations showed a positive correlation with *K. marxianus* and *C. inconspicua,* and a negative correlation with *C. jaronii* ($P < 0.05$), as can be observed in Figure 2B. *S. cerevisiae* was positively correlated with *Acetobacteraceae* and with *A. pasteurianus (*Figure 2B).

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

However, few statistically significant correlations were found in the H fermentations (Figure 3B); *A. pasteurianus* was found to be negatively associated with sucrose, while *Gluconobacter* was positively 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 the microbiota and volatilome profile. Significantly different associations were observed between the secondary metabolites and the main OTUs in the B and H fermentations, as shown in Figure 3 ($P < 0.05$). The main bacterial and fungal taxa in the B fermentations (Fig. 3A), that is, *S. cerevisiae, H. opuntiae, L. plantarum, A. pasteurianus, K. marxianus, C. inconspicua* and *L. fermentum* were statistically correlated with the key-aroma and fermentative markers, while the minor OTU bacteria (*Enterobacteriaceaeee, Trabulsiella, Erwinia,* and *Gluconobacter)* and *H. opuntiae* were statistically negatively correlated with the acids and phenols. In short, positive correlations were found between *S. cerevisiae* and ethyl octanoate, 2-methyl-butanal and 3-methyl-butanol, between *H. opuntiae* and 2-pentanol ($P < 0.05$), between *L. plantarum* and 2-heptanol, 2-methyl-butanal, 3-methyl-1-butanol and ethanol, and between *L. fermentum* and 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 ($P < 0.05$).

Fewer correlations were observed in the H fermentations than in the B fermentations (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 with 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 with benzaldehyde, acetoin, acetic acid, benzyl alcohol and β-phenylethylacetate ($P < 0.05$).

DISCUSSION

In this study, the changes that have taken place in the physico-chemical composition, microbial counts and microbiota diversity of cocoa beans in two different fermentation processes, that is, in boxes (B) and heaps (H), inoculated or not inoculated with yeasts as a starter culture, have been investigated. The ability of the survival and growth of selected starter strains, in this case *S. cerevisiae* ID67 and *T. delbrueckii* ID103, during cocoa fermentation is one of the most important features to ensure their effect during this process. These starter strains have shown the ability to coexist with autochthonous microbial communities in fermented cocoa beans. However, the yeast cultures used in the present study did not significantly modify the microbiological dynamics, physico-chemical parameters or metabolites produced during fermentation, whereas the same starter strains influenced the fermentative process and the quality of the end-products in at least one cocoa hybrid variety (9). It is important to note that the initial yeast load in a previous study was lower than those observed in our study and this might explain the discrepancies on the impact of the same yeast culture during cocoa fermentation. The different fermentation practices, the cocoa variety and the use of different starter cultures on site during cocoa bean fermentation play important roles in the success of the starter culture used during fermentation and might also explain the discrepancies found between studies (5, 6, 8–14). Our results confirmed that the performance of starter cultures on cocoa fermentation might change from the geographic origin. Moreover, the effectiveness of the cultures depends on the complexity of the microbial consortia. This in turn is influenced directly by the used fermentation method, each of which is characterized by its own micro-environment and is affected by oxygen availability, local agriculture practices, temperature, amount of cocoa mass used, etc.

During fermentation, cocoa beans constitute an ecological niche for a wide range of microbes. The advances made in studying the dynamics of cocoa microbial communities have

shown that the composition of these communities follows predictable patterns that report a rapid decline in yeast counts after 48 hours, when the sugars are depleted, a rise in temperature and an increase in LAB and AAB (15–19). The great impact on the microbial dynamics and succession during cocoa fermentation have been explained by considering 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 facilitated the detection of new yeast, LAB and AAB species. A restricted microbial population, that includes *H. opuntiae, A. pasteurianus* and *L. fermentum* , has already been reported for fermented cocoa beans and has also been detected in our study (8, 13, 20). However, some discrepancies can be observed among the most abundant microbial species in fermented cocoa beans, which may vary considerably from country to country. Through the application of amplicon-based sequencing in our study, we have been able to detect unusual yeasts, such as *C. jaroonii, Lasiodiplodia theobromae* and *Botryosphaera,* during cocoa fermentation, none of which had previously been detected. Noteworthy, there is a lack of information available regarding the incidence of minor microbial groups (21). In spite of the great advances made in microbial ecology, through next-generation sequencing, microbial species level identification and strain level differentiation still represent a challenge which needs to be addressed in the future to achieve an accurate identification.

This study, in an attempt to gain more knowledge about the range of potential interactions between microbial communities, describes a possible co-occurrence and co-exclusion. Our results, which were obtained from statistical correlations of bacterial ecology, LAB modulated the yeast culture, in agreement with previous observations (22). It should be noted that, in our study, these associations depended on the type of fermentation process, and the correlation dataset was used to explore the possible microbial dynamics, interactions and metabolism. This information can offer information about the kinetics of substrate consumption and aroma production by the microbiota present in fermented cocoa beans. However, it has

been found that the correlations depend on the number of samples in which a Type II error reflects the failure to reject a null hypothesis that is not true.

The dynamics of the non-volatile compounds has shown a successful competition for nutrients by the microbial populations within the cocoa fermentations. The ability of the fungal and bacterial communities to reduce sugars that has been observed in our results has been studied in detail and supported by previous studies (23, 24). As far as the organic acid dynamics is concerned, citric acid showed the highest concentration at the beginning of both fermentations and it then decreased over time. This utilization of citrate has been attributed to bacteria, which metabolizes it into acetic acid, carbon dioxide and lactic acid (25). However, not only can bacteria utilize citrate as an energy source, but some isolates within *Candida krusei* have also been reported to assimilate citrate during cocoa fermentation (26). This specific yeast was not detected in our study during cocoa fermentation, while the presence of the most abundant yeasts found in this study, that is, *H. opuntiae* and *S. cerevisiae*, have never shown the capability to assimilate citrate *in vitro* (27). Therefore, it has been hypothesized that the observed citrate assimilation was due to such LAB as the highly abundant *L. fermentum*, as also supported in a previous study (28). The high concentrations of succinic acid, from 48 hours to the end of the fermentation, is likely related to the metabolic activity of the LAB, since these bacteria have shown the capability to produce succinic acid from the citrate fermentation or convert fumaric and malic acids to succinic acid (29, 30). The reduction in pH in the pulp caused by LAB producing lactic acid favors the growth of AAB species, such as *A. pasteurianus*, which is capable of producing acetic and malic acids (29, 31).

Biochemical reactions play key roles 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, as did their composition. According to Kone *et al.*, (33), *P. kudriavzevii* and *S. cerevisiae* are the most important producers and contributors of cocoa aroma compounds, and these are 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 such yeasts as *S. cerevisiae, Candida,* and to other yeast species that have not been identified in this study on fermented cocoa beans (33–35). Apart from the production of VOCs by fungi, AAB are known to oxidize alcohols, such as ethanol, isoamyl alcohol and 2-phenylethanol, to produce acids and acetaldehydes (36, 37).

We observed that the main bacterial group found in our study increased the concentration of succinic, acetic, lactic acids, acetoin, alcohols, esters and acetaldehydes. Overall, the biochemical contribution to food ecosystems might change according to 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 has allowed us to obtain new insights into the microbial development and aroma formation that take place during cocoa fermentation. Here, we observed that the starter culture modulated the microbiota composition of fermented cocoa beans and only marginally affected the metabolites, which were influenced more by the type of process that was carried out. Accordingly, the difference found between Box and Heap fermentations might be explained by considering the environmental and processing conditions, in which the micro-environment of each process plays an important role. The application of the omics approach has confirmed that fermented cocoa beans have complex microbial communities that are dominated by restricted bacterial and yeast populations. Future research is needed to assess how fermentation methods, or the presence of the starter cultures, can affect the final characteristics of chocolate.

MATERIALS AND METHODS

Cocoa bean fermentations. The lyophilized *S. cerevisiae* ID67 and *T. delbrueckii* ID103 strains 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 mid-crop in 2016 (September-October 2016). The strains were chosen according to the study of Visintin *et al.*, (9). Briefly, cocoa pods of the *Forastero* hybrid were harvested by traditional methods and stored on the ground for 2-3 days before opening the pods. The cocoa pods were cut with non-sterile machetes and the beans and the adhering pulp were removed by hand. Approximately 3 h after breaking the pods, the cocoa bean-pulp was grouped into two independent lots (for the Box and Heap processes). Approximately 200 kg of fresh cocoa bean-pulp was used for the B fermentation; it was placed in a wooden box (0.06 m^3), covered with banana leaves and closed with a wooden lid to protect it from the open air. The heap fermentations were set up with smaller amounts of beans than the box fermentations, due to the fact that an adult can manually turn no more than 100 kg of bean-pulp. These beans were piled on top of banana leaves and covered with other banana leaves and jute rags. The field experiment involved inoculating the cocoa beans-pulp with *S. cerevisiae* ID67 (S) or with *S. cerevisiae* ID67 in co-cultures with *T. delbrueckii* ID103 (ST) in a 1:1 ratio (weight: volume) at the beginning of both fermentation processes (B and H). The lyophilized starter cultures were revitalized in a sterile saline solution for 30 min at room temperature and were progressively added and mixed with the cocoa-pulp mass to a final concentrations of 7.0 ± 0.2 Log CFU g^{-1}. Moreover, non-inoculated fermentations were carried out, without adding any starter culture to either fermentation process (B and H), and were used as a control. All the trials were performed in duplicate (n=12), according to the local agricultural practices: the cocoa bean-pulp mass was turned manually at 48 and 96 h and the fermentations were stopped after 120 h by spreading the beans on a drying platform. An aliquot of 1-1.5 kg of cocoa-pulp was collected in sterile bags after 0, 48, 96, and 120 h for each of the six experimental trials. The pulp was taken randomly from at least five different zones of the fermentative mass in both the B or H fermentations. It should be noted that sampling was performed at 48 and 96 h before mixing the mass. Approximately 20 g of sample was collected, stored at -20°C and transported,

441 on dry ice, to the Department of Agriculture, Forestry and Food Sciences (University of Turin,
442 Italy) for further metabolites analysis. Aliquots of 25 g of each sample were subjected to
443 microbiological analyses at an experimental laboratory that had been set up on site. The pH
444 values and temperatures were measured at the same sampling times during fermentation
445 considering an average of five random zones of the cocoa bean-pulp mass, and using a pH-
446 thermometer (Crison, Modena, Italy).
447
448 **Culture-dependent microbial community dynamics.** A classical microbiological
449 analysis was performed on samples recovered at 0, 48, 96 and 120 h. Twenty-five grams of
450 cocoa beans and the adhering pulp were homogenized with 225 mL of Ringer's solution (Oxoid,
451 Milan, Italy). Decimal dilutions were prepared in quarter-strength Ringer's solution. Aliquots
452 of 0.1 ml of the appropriate dilutions were spread in triplicate on the following media: WL
453 Nutrient agar (WLN; Lab M, Heywood, Lancashire, UK) plus 1 µg/mL of tetracycline (Sigma-
454 Aldrich, Milan, Italy) to count the total yeasts incubated for 3/5 days at 30°C, De Man Rogosa
455 and Sharpe agar (MRS, Oxoid, Milan, Italy) plus 2 µg/mL of natamycin (Sigma-Aldrich, Milan,
456 Italy) for the growing LAB, incubated at 30 °C for 48 h and Acetic Acid Medium (1 % glucose,
457 0.8 % yeast extract, 0.5 % bacteriological peptone, 15 g/L agar, 0.5 % ethanol, 0.3 % acetic
458 acid), plus 2 µg/mL of natamycin (Sigma-Aldrich, Milan, Italy) for the growing acetic acid
459 bacteria (AAB) incubated at 30 °C for 3/5 days. The results obtained from three independent
460 determinations were expressed as the means of Log CFU g^{-1}. Yeast colonies (5-8 for each
461 sampling point) were randomly isolated from the highest WLN dilution plate. These colonies
462 were further purified by streaking, and were then stored in 20 % v/v glycerol. A 1 ml aliquot
463 of the first 10-fold serial dilution was collected at each sampling and centrifuged at the
464 maximum speed for 30 s.
465
466 **Assessment of the yeast ecology by means of culture-dependent analysis.** DNA
467 extraction from single isolates was performed as described by Cocolin *et al.,* (39), and
468 normalized at 100 ng L^{-1}. Isolates were grouped in relation to their Restriction Fragment Length

469 Polymorphism (RFLP) profiles, which were obtained after enzymatic restriction of the
470 amplified ITS-5.8S rDNA region, as previously described by Korabečná et al., (2003). The
471 ITS-5.8S rDNA region of at least three representative isolates of each RFLP-group was used
472 for sequencing (GATC Biotech, Colonia, Germany). An REP-PCR assay was performed on all
473 the isolates previously identified as *S. cerevisiae* and *T. delbrueckii* , according to the procedure
474 outlined in a previous study by Visintin *et al.*, (2017) (9). A starter culture from the REP-PCR
475 profiles was compared with those of *S. cerevisiae* ID67 and *T. delbrueckii* ID103.
476
477 **Chemical analysis.** Fermented lyophilized bean-pulp samples (0.20 g) were washed
478 with 2 ml of pure hexane (Sigma-Aldrich, Milan, Italy) and vortexed for 5 min. The
479 homogenate was centrifuged (6000 x g, 4°C for 15 min) and the supernatant was removed. The
480 washing process was repeated twice, and the precipitate was dried after the washings and re-
481 suspended with 10 ml of a 70:29.5:0.5 acetone/MilliQwater/formic acid solution (Sigma-
482 Aldrich, Milan, Italy). The solution was vortexed, centrifuged and clarified by filtration through
483 0.45 µm syringe filters (Labware, LLG, CA, USA) and then evaporated. The extract was re-
484 suspended with 5 ml of MilliQ water and passed through a C18 cartridge (Sep-pack, USA). The
485 column was washed with 5 ml of MilliQ water to recover the samples.
486 The HPLC system (Thermoquest Corporation, San Jose, CA, USA) was equipped with
487 an isocratic pump (P1000), a multiple autosampler (AS3000) fitted with a 20 µL loop, a UV
488 detector (UV100) set at 210 nm and a refractive index detector (Spectra System RI-150, Thermo
489 Electro Corporation). The analyses of the sugars (glucose, fructose and sucrose) were
490 performed isocratically, at 0.6 ml min^{-1} and 80 °C, with a 300 × 7.8 mm i.d. cation exchange
491 column (Aminex HPX-87P) equipped with a Cation Carbo-P Microguard cartridge (Bio-Rad
492 Laboratories, Hercules, CA, USA). The analyses of the organic acids (acetic, lactic, malic,
493 succinic, oxalic, gluconic, tartaric, pyruvic, fumaric and citric acid) were performed
494 isocratically, at 0.8 ml min^{-1} and 60 °C, with a 300 × 7.8 mm i.d. cation exchange column
495 (Aminex HPX-87H) equipped with a Cation H+ Microguard cartridge (Bio-Rad Laboratories,
496 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 the samples of each biological replicate were analyzed in triplicate and the identification of compounds was performed by comparing the retention time of the standard. The calibration curves of the standards were obtained 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 the microbiota consortia. The dynamics of the volatile organic compounds (VOCs) of the fermented cocoa bean-pulp was obtained under different previously lyophilized conditions using the headspace solid phase micro-extraction (HS- SPME) technique, in which the fiber conditions and oven temperatures were set as previously described by Rodriguez-Campos *et al.,* (2011) with some modifications (41). Samples of each biological replicate were analyzed in triplicate. The analysis was conducted in a 20 ml vial filled with 2 ml of 20 % NaCl and 0.1 g of the sample and 10 µl of 5-nonanol in ultrapure water was added to each sample at a 50 mg/L concentration as an internal standard for the semi-quantification. The fibers with VOCs were injected into the Gas Chromatography-qMass Spectrometry (GC-qQP2010 Plus, Shimadzu, USA), which was 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) and helium was used, at a constant flow rate of 1 ml/min, as the carrier gas. The detection was carried out by means of the electron impact mass spectrometer in total ion current mode, using an ionization energy of 70 eV. The acquisition range was set at *m/z* 33-350 amu. The peaks were identified by comparing the mass spectra of the peaks with the spectra of the MIST05 library and through a comparison of the retention indices (a matrix of a homologous series of C8-C24 was used) with an injected pure standard according to 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-effect models for a non-normally distributed data set. Mixed models were chosen because of 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 and, 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 according to Crawley (43) using R version 3.3.2. The assessment of the mean difference between the box and heap fermentations over a specific fermentation period was subjected to a t-test, in which each fermentation condition was compared between fermentation methods (B and H). In addition, Spearman's correlation test was used to assess the correlations between the OTUs and to establish any changes in concentration over the fermentation period.

DNA extraction, library preparation and sequencing. The total DNA was extracted from pellets of the cocoa matrices using a MasterPure Complete DNA & RNA Purification kit (Illumina Inc, San Diego, CA), according to the manufacturer's instructions. Bacterial communities were studied by amplifying the V3 and V4 regions of 16S rRNA using the primers and under the conditions described by Klintword *et al.* (44). The 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 using a 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 in which the weight of the library, measured by means of Qubit Fluorometric Quantitation

553 (Thermo Fisher Scientific), and the mean amplicon size were taken into account. A denaturated
554 20pmM pool was obtained by mixing 5µl of NaOH 0.2N with 5 µl of the 4 nm pool. A final 10
555 pM library was combined with 10% PhiX. Sequencing was performed using an MiSeq
556 instrument (Illumina) with V3 chemistry, according to the manufacturer's instructions, and
557 250-bp paired-end reads were generated.
558
559 **Bioinformatics.** The thus obtained paired-end reads were first assembled with FLASH
560 software (46), with default parameters. The joint reads were further quality filtered (Phred <
561 Q20) using the QIIME 1.9.0 software (47). Reads shorter than 250 bp were discarded using
562 Prinseq. For the 16S data, the OTUs were picked at a 99 % of similarity threshold, and centroid
563 sequences of each cluster were used to assign the taxonomy by mapping against the Greengenes
564 16S rRNA gene database, version 2013, as recently described (48). The chloroplast and
565 mitochondria sequences were removed from the dataset. For the ITS dataset, 97 % of similarity
566 was picked for the OTUs, by means of UCLUST clustering methods (49), and representative
567 sequences of each cluster were used to assign the taxonomy using the UNITE rDNA ITS
568 database, version 2012, by means of the RDP Classifier. Weighted and Unweighted UniFrac
569 distance matrices, as well as the OTU table, were used to find differences between the
570 fermentation processes (B and H) and under different conditions (inoculated and non-
571 inoculated) in the Adonis and Anosim statistical test in the R environment in order to avoid
572 biases due to different sequencing depths. All the samples of each dataset were rarefied at the
573 lowest number of reads after raw read quality filtering. QIIME was used to produce a filtered
574 OTU table at 1 % in at least 2 samples. The OTU table displays the highest taxonomy resolution
575 reached when the taxonomy assignment was not able to reach the species level, or when the
576 genus or family name was displayed. The Kruskal–Wallis statistical package and Mann–
577 Whitney tests were used to find significant differences ($P < 0.05$) in the microbial taxa
578 abundance profiles and in the Shannon-Wiener diversity index *H'*, according to the time,
579 conditions and methods. Spearman's rank correlation coefficient was obtained as a measure of
580 the association between the microbial OTUs that occurred in at least 2 samples and the chemical

variables through the *psych* function and plotted through the *corrplot* package of R. The OTUs that occurred in at least 2 samples of the microbial communities were conglomerated, by means of hierarchical clustering analysis, using Ward's method, which was acquired thorough the *heatplot* function plotted by the *made4* package of R.

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

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723

TABLE LEGEND

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

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

Table 3. Classification at a family / genus level of the occurrence of the bacterial taxonomic groups, achieved by means of amplicon sequencing, expressed as relative abundances. Only OTUs with an incidence above 1 % in at least 2 samples are shown

738 **FIGURE LEGEND**

739

740 **Fig 1.** Dynamics of the sugar and organic acid compounds in the cocoa bean-pulp inoculated
741 and non-inoculated during Box and Heap fermentations expressed as mg/g. Data are expressed
742 as mean ± SD values from triplicate determinations. **A)** sucrose, **B)** glucose, **C)** fructose, **D)**
743 citric acid, **E)** succinic acid, **F)** malic acid, **G)** acetic acid, **H)** gluconic acid and **I)** lactic acid

744

745 **Fig 2.** Spearman's correlation between the microbial OTUs observed with an incidence above
746 >1 % in at least 2 samples. The samples are labeled according to the fermentation method **A)**
747 Box and **B)** Heap. Rows and columns are clustered by means of Ward's linkage hierarchical
748 clustering. The intensity of the colors represents the degree of correlation between the fungal
749 and bacterial OTUs, as measured by Spearman's correlation. The intensity of the colors
750 represents the degree of correlation between the yeast and bacteria, where blue represents a
751 negative degree of correlation and red a positive degree of correlation.

752

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

760

761

762

763 **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}

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

767 **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

768 Values are expressed as the mean of duplicate determinations. The abundance of OTUs in the 2 biological replicates of each sampling time was averaged. Samples are labeled according to the fermentation period (0,

769 48, 96 and 120 h), fermentation method (Box and Heap) and condition (inoculated with S, ST and non-inoculated).

770

771 **Table 3.**

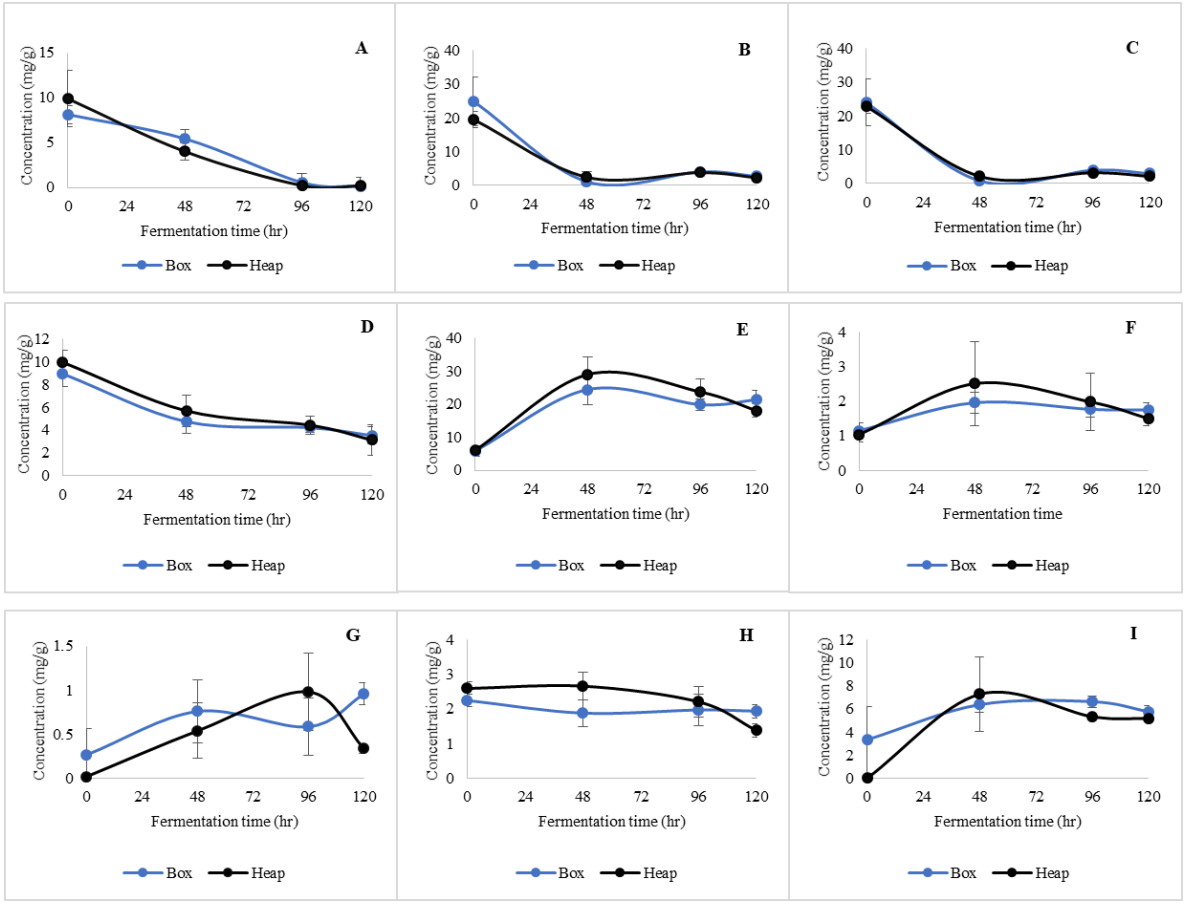
	Box				Heap				Box				Heap				Box				Heap			
	T0	**T48**	**T96**	**T120**	**T0**	**T48**	**T96**	**T120**	**T0**	**T48**	**T96**	**T120**	**T0**	**T48**	**T96**	**T120**	**T0**	**T48**	**T96**	**T120**	**T0**	**T48**	**T96**	**T120**
	S. cerevisiae								***S. cerevisiae + T. delbrueckii***								**Non-inocualted**							
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

772 Values are expressed as the mean of duplicate determinations. The abundance of OTUs in the 2 biological replicates of each sampling time was averaged. Samples are labeled according to the fermentation period (0,

773 48, 96 and 120 h), fermentation method (Box and Heap) and condition (inoculated with S, ST and non-inoculated)

774

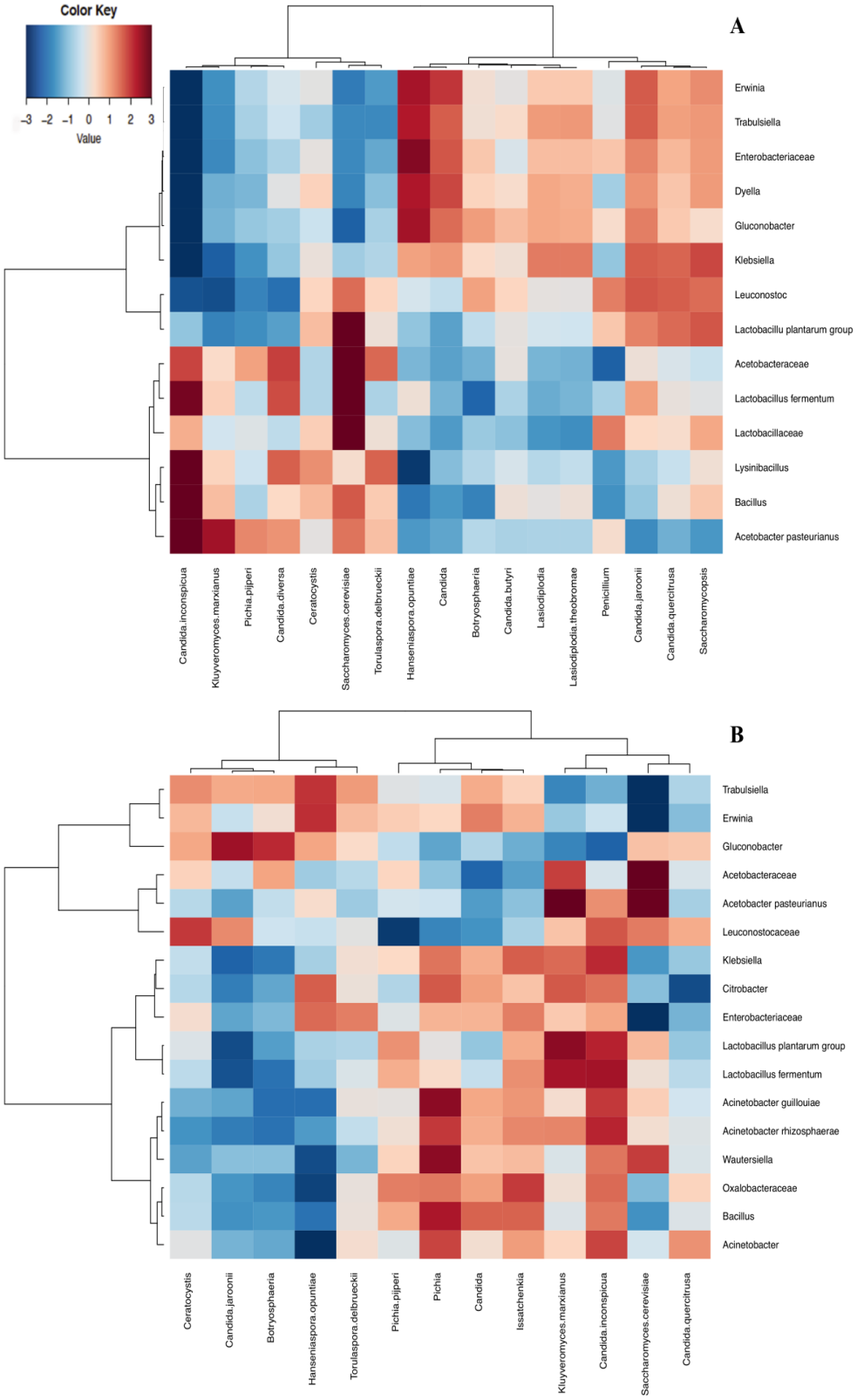
775 **Fig. 1**



776

777

Fig. 2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Dyella
Gluconobacter
Klebsiella
Leuconostoc
Lactobacillu plantarum group
Acetobacteraceae
Lactobacillus fermentum
Lactobacillaceae
Lysinibacillus
Bacillus
Acetobacter pasteurianus
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Kluyveromyces.marxianus
Pichia.pijperi
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Saccharomyces.cerevisiae
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Hanseniaspora.opuntiae
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Botryosphaeria
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Candida.quercitrusa
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Erwinia
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Leuconostocaceae
Klebsiella
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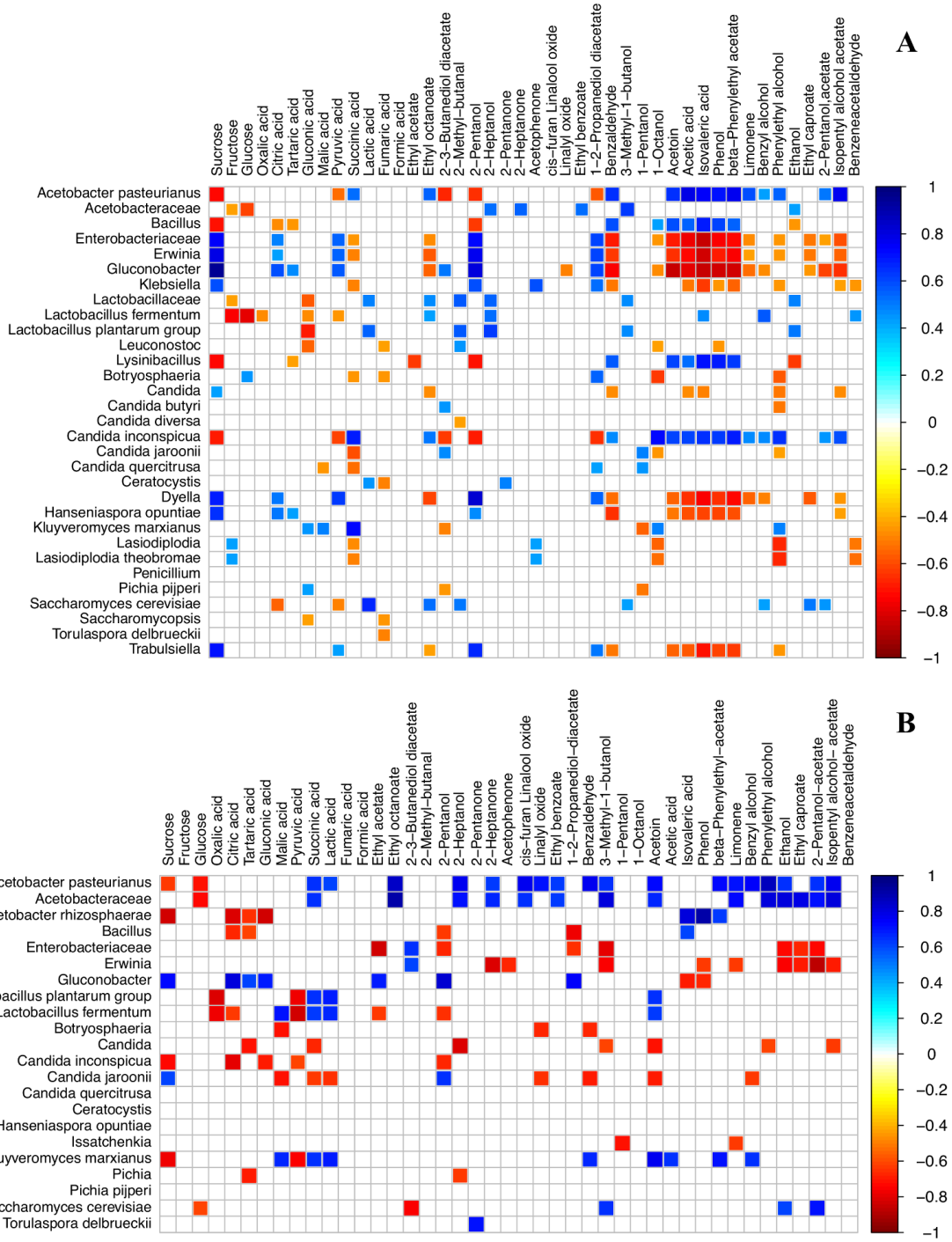
Fig. 3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Bacillus
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Gluconobacter
Klebsiella
Lactobacillaceae
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Lactobacillus plantarum group
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Lysinibacillus
Botryosphaeria
Candida
Candida butyri
Candida diversa
Candida inconspicua
Candida jaroonii
Candida quercitrusa
Ceratocystis
Dyella
Hanseniaspora opuntiae
Kluyveromyces marxianus
Lasiodiplodia
Lasiodiplodia theobromae
Penicillium
Pichia pijperi
Saccharomyces cerevisiae
Saccharomycopsis
Torulaspora delbrueckii
Trabulsiella
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Acinetobacter rhizosphaerae
Bacillus
Enterobacteriaceae
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Gluconobacter
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Lactobacillus fermentum
Botryosphaeria
Candida
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Candida jaroonii
Candida quercitrusa
Ceratocystis
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Issatchenkia
Kluyveromyces marxianus
Pichia
Pichia pijperi
Saccharomyces cerevisiae
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